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CHARACTERISATION OF BOVINE MHC CLASS I GENES

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ABSTRACT OF THESIS

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Polymorphism of bovine MHC (BoLA) class I molecules has been characterised mainly by serology. The use of alloantisera has led to the identification of 50 different serological specificities, most of which behave as alleles of a single highly polymorphic locus. However, molecular biological and biochemical studies suggest that there are at least two class I genes expressed from any one haplotype. The actual number of expressed MHC class I genes in cattle is as yet unknown. The aim of this study was to isolate different functional class I genes from a heterozygous animal, class I typed as BoLA- A10/A11. In order to investigate the expression and function of individual class I products, transfection and characterisation of a series of bovine class I genomic and cDNA clones isolated from this animal were performed.

From a group of fifteen bovine class I genomic clones, five different clones (1.3, 4.2, 15.2, 17.3x and 19.1), characterised by their exon 2 sequences and by restriction analysis of an amplified 3.2kb class I gene fragment, were transfected into mouse L cells. Of the five clones transfected, only 19.1 and 4.2 showed expression at the L-cell surface by using a murine monoclonal antibody specific for a non-polymorphic determinant on bovine MHC class I (IL-A88) in flow cytometry. The phage clone 19.1 which had been previously shown to express a bovine class I molecule with A11 specificity, served as a control in these transfection experiments. The transfectants obtained from both expressing clones were characterised serologically, biochemically and by cellular assays and both were found to encode A11 serological specificity. Interestingly, the products of the 19.1 and 4.2 transfected class I genes were indistinguishable by isoelectric focusing, despite having clearly different nucleotide sequences.

The class I gene from the 19.1 phage clone was subcloned into pBR322 to make pBoLA-19 which was used to determine its sequence. This plasmid transfected at a very high frequency with more than 90% of HAT resistant colonies positive for class I expression in the primary transfection. From the sequencing of the 19.1 gene and these transfection results it was clear that pBoLA-19 contains all the upstream regulatory and promoter sequences necessary for class I expression. The lack of expression from the three phage clones 1.3, 15.2 and 17.3x suggested that these clones might contain truncated class I genes or pseudogenes. In order to test their ability to be expressed, these clones were reconstructed by replacing the coding region of pBoLA-19 with the corresponding gene fragment from three non-expressing clones. For this the pBoLA-19 construct was modified into a class I gene expression vector (pBoLA-21). Plasmid pBoLA-21 carries the 5' and 3'-ends of 19.1 class I genomic clone, flanking a unique *Eag* I site which was used for subcloning PCR-amplified class I gene fragments. The exon 2 to 3'-untranslated region fragments were generated using primers based on sequences conserved in exon 2 and in the 3'-untranslated regions of the published bovine class I sequences. Both the 19.1 and 4.2 clones were reconstructed with this approach and expressed on transfection. The three non-expressing phage clones did not express even after reconstruction, suggesting that they are pseudogenes.

The potential of the pBoLA-21 vector for cDNA cloning and expression was tested by subcloning the bovine class I cDNA clone pBoLA-1, which is truncated at both the ends. The transfected cell line from pBoLA-1 showed detectable levels of class I molecules using IL-A88. Since both of the expressible class I genomic clones isolated from this animal encoded A11 specificity, a cDNA approach was adopted for the cloning of sequences representing possible A10 genes from this haplotype. A 1.2kb cDNA was PCR-amplified using a selective primer based in the 3'ut region of the class I gene, and the PCR product was subcloned into pBoLA-21 to test its expression. The transfectant obtained was recognised by IL-A88 and IL-A34 (an A10-specific mAb) indicating that the transfected cell line expressed a component of the A10 haplotype.

To correlate the serological specificities of the individual expressed products with nucleotide sequences, all three expressing genes were sequenced. The new class I sequences from pBoLA-19, pBoLA-4 and pBoLA-10 were compared with each other and the other published sequences in order to establish whether these sequences represented products of single locus or genes representing different loci. It appeared from the sequence analysis that all three sequences isolated from this animal are likely to represent different loci. The approach used in this project will facilitate the functional analysis of individual locus products, and will allow the number of expressed class I loci to be resolved.

DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for a degree. The work on which it is based is my own except where stated in the text and in acknowledgement section.

Suminder Mohan Sawhney

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Dedicated to my mother and father

List of Abbreviations:

a.a	amino acid
ATP	adenosine triphosphate
AP	ammonium persulphate
bp	basepair
ABS	antigen binding site
APC	antigen presenting cell
B-LCL-271	B-lymphoblastoid cell line 721
BoLA	bovine lymphocyte antigen
β_2m	β_2 -microglobulin
cDNA	DNA complementary to mRNA
CIP	calf intestinal phosphatase
CPL	compartment for peptide loading
CTL	cytotoxic T lymphocytes
dNTP	deoxy nucleoside triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetra-acetic acid
ER	endoplasmic reticulum
FACS	fluorescent activated cell scanner
HBSS	hank's balanced salt solution
HLA	human leucocyte antigen
IAA	isoamyl alcohol
1D	one dimensional
IEF	isoelectric focusing
Ig	immunoglobulin
kb	kilobase pairs
kDA	kilodalton
L	litres
LB	Luria broth
M, mM, μ M	molar, milimolar, micromolar
μ l, μ g, ml	microliters, microgrammes, mililiters

MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
mRNA	messenger RNA
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pTK	Thymidine kinase gene
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	tetramethylene diamine
UT	untranslated
UV	ultraviolet
UWGCG	University of Wisconsin Genetics Computer group
X-GAL	5-Bromo-4-chloro-3-indoyl- β -D-galactopyranoside

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Chapter 1

LITERATURE REVIEW

1.1 Introduction

Lymphocytes are the agents of antigenic specificity in the immune response. They are divided into two main groups: B cells that make immunoglobulins (Ig), and T lymphocytes that participate in almost all antigen-specific immune response. The mechanism of antigen recognition by T lymphocytes has evolved to allow an organism to define self and yet also to distinguish a sufficient number of non-self antigens so that it can be protected from life threatening pathogens. A proliferative response by T cells is triggered only when receptors on its surface interact with antigen presented on the surface of the target cell. Fundamental to a T cell encounter with any antigen is the interaction of major histocompatibility complex (MHC) molecules with T cell receptor (TcR). The MHC molecules bind an antigenic peptide, thereby forming a composite ligand recognised by the TcR.

There are two structurally distinct, but related, families of MHC molecules, class I and class II, that present antigen to two subsets of T cells: class I MHC molecules present antigens to T cells that express the CD8 cell surface glycoprotein, and class II MHC molecules present antigen to CD4 T cells. One feature of MHC molecules that distinguishes them from any other family of molecules is the high degree of polymorphism maintained in both classes of molecules. Polymorphism in the MHC has a profound effect on the extent and quality of the immune response, and provides a better capacity to respond to a wider variety of antigens (Kappes and Strominger, 1988; Lawlor et al. 1990). Between individuals, the variability in the response to an antigen can be due to the variable capacity of the MHC products to bind to a particular peptide. Thus, the MHC plays a vital role in the immune response and it

is therefore important to investigate its features.

The major histocompatibility complex (MHC) is one of the best characterised areas of the mammalian genome where a set of linked genes encoding cell surface and plasma proteins that show various fundamental functions in the immune system. The MHC is best known in humans and mice where it is divided into three areas : class I, class II and class III (Klein, 1986; Campbell and Trowsdale, 1993). The class I area is further sub-divided into class Ia and class Ib (Klein, 1986). The human major histocompatibility complex (MHC), also referred to as HLA, resides on the short arm of chromosome 6 (Spring et al. 1985) and spans four million base pairs (Mbp) of DNA. In mice, the MHC is named the H-2 complex and is located on chromosome 17. A description of the discovery and molecular elucidation of these loci now follows:

1.2 History of the MHC

The first indications of the existence of the MHC were provided by Gorer (1936). A year later, he reported on hereditary genetic differences in the blood of mice, and this blood group antigen appeared to be involved in the rejection of transplanted tumors in mice. Ten years later, a study on the phenomenon of graft rejection in congenic mouse strains resulted in the definition of a set of genes coding for cell surface molecules that determined tissue compatibility (Gorer et al. 1948).

The molecules involved in the process of tissue rejection were called histocompatibility antigens and a set of loci coding these molecules in mice was called the H-2 complex (Snell, 1948). These loci elicited a specific antigenic response (by generation of antibodies) and all mapped to chromosome 17.

Characterisation of these loci, by immune sera generated from tumor cells, from inoculation of lymphoid tissue and by skin grafting, and their subsequent mapping, was aided by the use of inbred (genetically identical) and congenic (genetically identical except for a region of chromosome of interest) strains of mice, (Klein, 1975). Subsequent use of these strains led to the mapping of the serological reactions to the region of chromosome, giving rise to the concept of two loci (K and D), controlling tissue transplantation in mice (Klein and Shreffler, 1972). The existence of the third locus, the L locus, was proposed to explain anomalous antibody typing results in different strains of mice (Snell, 1974).

The exact role of these molecules did not become apparent until Zinkernagel and Doherty (1974) reported the MHC-restricted T cell response against viral antigens. They proposed that the CTL had dual specificity and that they recognised viral antigens as well as MHC molecules expressed on the same target cell. This formed the basis for all further research on MHC-restricted T cell responses, which led to the definition of the role of MHC products in antigen presentation. The class I loci capable of acting as MHC restriction elements (class Ia) are functionally distinct from another group of class I loci (class Ib). The class Ib loci of mouse have been subdivided into four subfamilies, on the basis of chromosome location and sequence H-2Q, T and M encoded on distal half of the mouse MHC chromosome 17 (Stroynowski, 1990) and CD1 on chromosome 3 (Calabi and Bradbury, 1991).

1.3 Discovery of class I loci in man

Soon after the description of a genetic system involved in graft rejection in the mouse, indications of a similar system in humans were found (Gibson and Medawar, 1943). They observed a graft rejection process in which the host became sensitized

by the first graft, and therefore a second graft was rejected more rapidly. However, in contrast to fast developments in the definition of the mouse MHC, it was not until the beginning of the sixties that research on the human transplantation antigens (human leucocyte antigens, HLA) started. From then on, the characterization of the HLA system developed rapidly.

The human leucocyte antigens were first described by J. Dausset, R. Payne and J. van Rood in 1958 (Klein, 1986), and were subsequently named HLA. These were described as highly polymorphic (Dausset, 1981) and mapped to chromosome 6 (Van Someron et al. 1974). The investigation of these antigens has relied on different methods of generating sera than were used in mice. Most antibodies for the analysis of the HLA system have been derived from a) multiparous women, b) people who have received multiple blood transfusions and c) volunteers who have undergone reciprocal skin grafts. Depending upon differences in HLA type and the degree of antigenic stimulation, these antisera can be of sufficiently high titre and specificity to be used as tissue typing reagents. These studies have led to the classification of three classical class I loci, which are called HLA-A, HLA-B and HLA-C (Klein, 1986).

With the increasing knowledge of the HLA system, research on the MHC of other species has also developed. At present, homologues of the human and murine MHC have been detected in some twenty vertebrate species, including cattle. New molecular techniques such as DNA cloning have also been used to uncover a multitude of new genes/polypeptides related to classical class I and class II MHC sequences.

1.4 Structure of the MHC class I and class II molecules

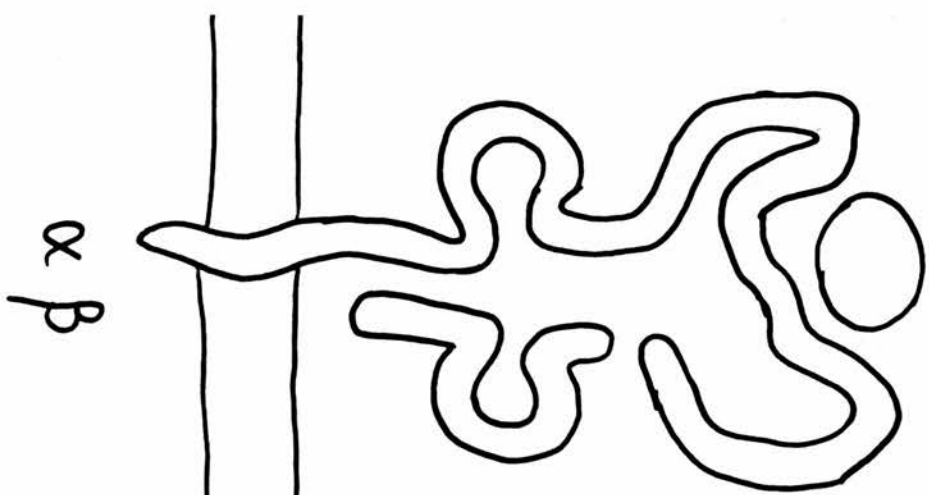
MHC molecules are membrane bound glycoproteins. They occur at the surface of expressing cells as heterodimers, composed of two non-covalently associated chains. According to difference in their structure, tissue-specific expression and function, two categories of MHC molecules are distinguished: class I and class II molecules (Fig 1.1).

1.4.1 *Class I molecules*

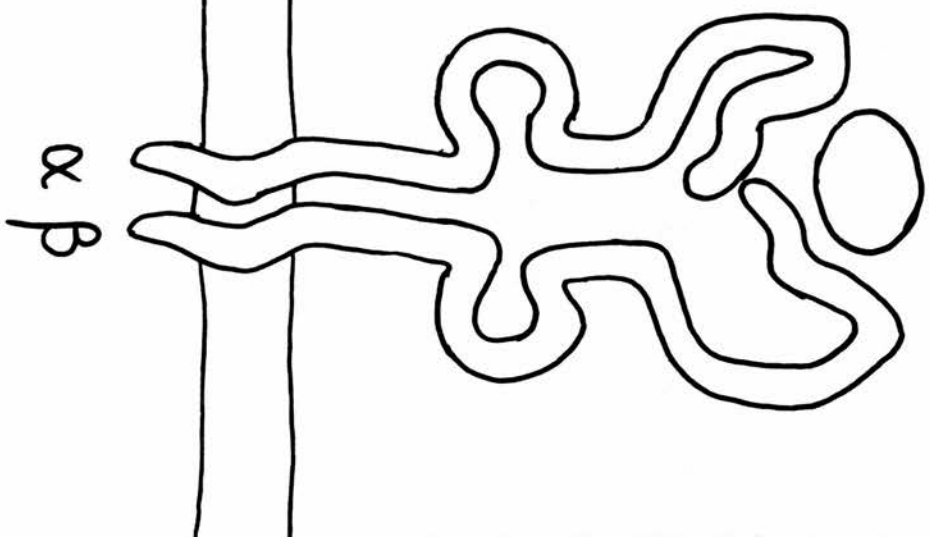
Class I MHC molecules, found on most cells, are composed of a 44kD integral membrane α chain associated with a non-MHC-encoded nonpolymorphic 12kD light chain known as β_2 -microglobulin (β_2m). Broadly, the class I α chain can be divided into three regions: an amino-terminal extracellular domain, hydrophobic transmembrane region and carboxy-terminal cytoplasmic region. The details of this organisation are described below :

The extracellular portion of the class I proteins can be sub-divided into three domains (α_1 , α_2 and α_3) comprising amino acid residues 1-90, 91-180 and 181-271 (Ploegh et al. 1981). The membrane-distal domains (α_1 and α_2) are extremely polymorphic and form the unique antigen binding region of the class I molecule, whereas the α_3 domain is relatively conserved (Michaelson, 1983). The first domain (α_1) has an asparagine-linked carbohydrate attachment site at amino acid 86 in mouse and human class I molecules.

Fig. 1.1 Schematic representation of the structure of class I and class II molecules.



Class I



Class II

The second domain (α_2) has a second carbohydrate attachment site at amino acid 176 in H-2 class I molecules, but not in HLA class I molecules (Nathenson et al. 1981). Cysteines capable of forming internal disulphide bridges occur at amino acids 101 and 164 in the second domain and at 203 and 259 in the third domain (Kimball and Coligan, 1983) and allowed the delineation of the protein into three separate domains. The α_3 domain associates non-covalently with β_2m on the cell surface (Yokoyama and Nathenson, 1983), which stabilizes the extracellular expression of the molecule. Indeed, H-2D^b and H-2L^d molecules which do not associate efficiently with β_2m , were detected in small quantities on the cell surface (Maloy et al. 1980; Coligan et al. 1980). The membrane proximal extracellular domains (α_3 and β_2m), resemble the C domains of immunoglobulin molecules (Tragardh et al. 1979). The α_3 domain plays an important role in interacting with class I restricted T cells expressing the CD8 glycoprotein (Parnes, 1989). This role arises from juxtaposition of the Ig- like domains, which are thought to be an adaptation for binding of the CD8 α chain, with α_3 residue 227 having the most effect on binding (Salter et al. 1990; Potter et al. 1989). This domain supports the peptide binding domains (α_1 and α_2) and connects them to the transmembrane anchor and short cytoplasmic domain of the class I α chain. The membrane binding region was localised to a hydrophobic stretch of residues (284-307) in H-2K^b (Nathenson et al. 1981). Generally, the membrane binding portion is approximately 24 amino acids long in HLA and H-2 class I molecules, and contains no charged or polar residues (Ploegh et al. 1981). This number of residues is enough to allow it to span the lipid bilayer of a cell if an α -helical configuration is assumed (Ploegh et al. 1981). Immediately outside the lipid bilayer there are basic residues which are postulated to help anchor the molecule in the cell membrane, by interacting with cytoskeletal elements. The cytoplasmic region is 39 amino acids long in H-2 molecules (Nathenson et al. 1981), and 31

amino acids long for HLA (Robb, 1978). This region has a high polar amino acid content (up to 50%) and contains the phosphorylation consensus sequence (Ser-Asp/Glu-Xaa-Ser(p)-Leu) which was identified in H-2 class I (Rothbard et al. 1980) and HLA class I (Pober and Strominger, 1981). The phosphorylation site is well conserved in the class I molecules of many species (Guild and Strominger, 1984).

Overall when comparing H-2 and HLA class I molecules in the transmembrane and cytoplasmic regions of the molecule there is marked divergence. This lack of homology suggests a general lack of structural constraint on these regions, in that they may only need to remain hydrophobic and hydrophilic respectively (Ploegh et al. 1981; Kimball and Coligan, 1983).

1.4.1.1 *X-ray crystallographic structure*

Huge advances have been made in recent years in determining the detailed structure of the class I molecule and in particular the structure of the antigen binding site. The three-dimensional structure of three human MHC class I molecules (HLA-A2, HLA-A68 and HLA-B27) have been elucidated by X-ray crystallography studies and currently serve as prototypes for all class I molecules (Bjorkman et al. 1987a; Garret et al. 1989; Madden et al. 1991).

The HLA-A2 molecule is composed of two sets of structurally homologous domains. The α_3 and β_2m are proximal to the membrane and are folded into β -sandwich structures (resembling the Ig constant domains), each composed of two antiparallel β pleated sheets connected by internal disulphide bonds. The membrane distal domains (α_1 and α_2) form a platform composed of eight anti-parallel β -pleated sheets, topped by a pair of α -helices with a long groove between the helices. The

four β -strands from each domain combine to produce a planar structure that forms the floor of the peptide-combining site (Bjorkman et al. 1987b), and each domain also contributes one of the two α helices which form the sides of the groove. This cleft, between the two helices, has now been shown as the key functional domain of MHC molecules for binding of antigenic peptides. The structures of HLA-A2 and HLA-A68 provided details of the peptide groove structure (Saper et al. 1991; Garret et al. 1989). The structure of the third class I molecule HLA-B27 revealed a clearer picture of peptides in the groove, indicating that peptides are bound in an extended conformation, with tight binding at either end of the groove between the peptide and conserved MHC residues (Madden et al. 1991; Madden et al. 1992). As the groove of class I molecules is closed at both ends, the length of peptides bound is restricted in size to 8-10 amino acids (Falk et al. 1991). Longer peptides can be accommodated by bulging in the middle (Guo et al. 1992). Six pockets, or subsites, designated A- F (Saper et al. 1991) were identified within the groove, suggesting that polymorphism, by creating and altering such sites, can influence the range of peptides bound by class I alleles. Various workers have used mutant class I molecules to evaluate the role of these pockets (Winter et al 1991; Buxton et al. 1992; Matsui et al.1993). Overall, all these studies demonstrated that peptide binding pockets play an important and distinct role in the binding of peptides and recognition of class I molecules by alloreactive CTLs.

1.4.1.2 Assembly of class I molecules

The newly translated heavy chains are guided to the endoplasmic reticulum (ER) by classical amino terminal signal sequences which are cleaved off once the molecules are co-translationally translocated across the ER membrane (Ploegh et al. 1979). The assembly of MHC class I molecules begins in the ER after β_2m associates with

class I heavy chain. Various workers have proposed a stepwise series of events in the assembly of class I molecules (Townsend et al. 1990; Elliott et al. 1991, 1992a). In one proposed pathway, the heavy chain (HC) can form a loose association with β_2m , which is then stabilized by peptide occupying the binding site. Alternatively, a short, high affinity peptide might stabilize a specific HC conformation, which can then associate with β_2m (Elliott et al. 1992b). The role of β_2m in class I assembly has been investigated by various workers in human mutant cell lines lacking β_2m , which suggested that β_2m -free class I molecules were not exported from the ER to the cell surface (Kragel et al. 1979; Koller et al. 1990; Ploegh et al. 1979). However, H-2D^b and H-2L^d molecules were detected on the cell surface of mouse mutant cell lines defective in β_2m expression, although the presence of β_2m may still influence the stability or the expression levels of these molecules (Kragel et al. 1979; Allen et al. 1986; Williams et al. 1989). These molecules were not recognised by D^b and L^d specific CTLs suggesting the conformation of these molecules were different from the native molecules. It was concluded from the above studies that although β_2m does not contribute to peptide binding directly, it is considered essential for correct cell surface expression of peptide /MHC class I complex as seen by CD8+ T cells (Vitello et al. 1990). Contrary to the above reports, Glass and co-workers (1992) reported that MHC class I restricted T cells can recognise targets from β_2m - deficient mice. Recently, Zugel et al. (1994) have also demonstrated the presentation of exogenous and endogenous peptides to CD8 cells in the absence of β_2m . It was found that specific short peptides, 9-10 amino acids (a.a.) in length, can induce folding of the heavy chain in the absence of β_2m . Both short and longer peptides (15a.a.) can stabilize preformed low-affinity heterodimers (Silver et al. 1991; Ortiz-Navarrete and Hammerling, 1991). Fahnestock and co-workers (1992), suggested that secreted empty class I molecules

are immunologically reactive and structurally similar to peptide-carrying class I molecules. However, class I molecules with peptides appeared to be more stable than empty class I molecules. The class I molecules are transferred from ER to the trans-golgi reticulum (TGR) where they are glycosylated before they are transported to the cell surface (Parham et al. 1977).

1.4.1.3 Role of calnexin

The efficient assembly and transport of class I molecules to the cell surface is critically dependent on the presence of suitable peptides. Inhibition of transport of peptide-free class I heavy chains appears to be due to interactions with an endoplasmic reticulum resident membrane protein called calnexin (p88; Degen et al. 1992). The ER retention of class I molecules serves as an example of quality control since it ensures that only fully assembled complexes reach the cell surface. Failure to achieve ER retention of class I molecules would upregulate expression of incompletely assembled class I molecules on the cell surface which could bind exogenous peptides, and lead to the recognition and destruction of normal cells by CTL (Schumacher et al. 1990; Ortiz-Navarrete et al. 1991). Calnexin associates with free class I heavy chains soon after synthesis, but this association is transient and calnexin dissociates from completely assembled class I complexes (Galvin et al. 1992). Degen and co-workers (1992) proposed that both β_2m and peptide were required for the dissociation of class I HCs from calnexin. Recently, Sugita and Brenner (1994) have shown that dissociation of class I heavy chain from calnexin is only β_2m dependent and peptide binding is not required. In addition to its role in intracellular retention, calnexin also protects the heavy chains from rapid intracellular degradation (Jackson et al. 1994).

1.4.1.4 Antigen processing

The production of most of the peptides destined for presentation by class I molecules begins in the cytosol, with the limited hydrolysis of antigenic proteins. Goldberg and Rock (1992) were first to report that the proteasome, a multicatalytic complex in the cytosol, played a vital role in generating peptides from cytoplasmic proteins. The involvement of this system in antigen presentation was further supported by the finding that two proteasome subunits (LMP-2 and LMP-7), are encoded by genes in the MHC adjacent to those of TAP (transporter associated with antigen processing). However, functional studies using cells deficient in expression of these proteasome subunits did not significantly impair the processing and delivery of antigens to class I molecules (Momburg et al. 1992; Arnold et al. 1992). These studies revealed that LMP-2 and LMP-7 are not essential for MHC class I presentation. Subsequently, other groups have shown that proteasomes may generate peptides with COOH termini that are appropriate for binding to class I (Driscoll et al. 1993; Gaczynska et al. 1993), and these may be trimmed in the ER at their amino termini, as suggested by Falk et al. (1990). Recently, it has been reported by two different groups that proteasome mediated proteolysis is probably the major source of class I presented peptides due to their endopeptidase activity (Rock et al. 1994; Dick et al. 1994). Rock and co-workers (1994) showed that when mouse cells were treated with Proteasome inhibitors (peptide aldehydes) there was a block in the assembly of the class I molecules.

1.4.1.5 Role of TAP in antigen transport

The discovery of ATP-dependent transporters encoded by TAP genes within the class II region of human, mouse and rat MHC (Monaco 1992; Trowsdale 1990; Powis et al. 1992b), immediately stimulated the speculation that they played a role in

translocating peptides from the cytoplasm to the lumen of the ER (Shephard et al. 1993; Neefjes et al. 1993). Transport of peptides from the LMP complex to the transporter, which resides in the ER membrane, was thought to be mediated by direct physical interaction between the two structures, or by simple diffusion. According to one hypothesis, these peptides are bound and protected from complete degradation by a chaperone, Hsp70, before reaching TAP (Srivastava et al. 1994). The involvement of TAP in peptide transport was suggested by the observation that cell lines defective in expression of TAP, such as murine RMA-S and human .174 and T2, do not efficiently express MHC class I molecules at the cell surface, but retained them in the ER (Spies et al. 1992; Kelly et al. 1992). Gene transfer experiments in these mutant cell lines demonstrated that the lack of class I surface expression and antigen presentation problems could be corrected by transfecting TAP1 and TAP2 cDNAs (Spies and DeMars, 1991; Attaya et al. 1992; Powis et al. 1991). These studies provided strong evidence that TAP mediates peptide translocation into the ER. This was also supported by the work of Van Kaer and co-workers (1992) who showed that mice deficient in LMP-2 gene expressed normal levels of class I molecules, whereas TAP 1 deficient mice showed a 20 fold reduction in the levels of class I surface molecules.

Polymorphism in the TAP gene results in an alteration of the spectrum of class I bound peptides (Powis et al. 1992a, 1992b), providing further indirect evidence for the role of TAP in peptide translocation. It has been suggested that TAP may function as a molecular ruler, selecting peptides of appropriate length with a suitable carboxy terminal residue for binding to a class I molecule (Germain, 1994; Momburg et al. 1994; Neefjes et al. 1993). The ability of TAP to transport peptides with different carboxy termini varies from species to species. In humans, TAP is

permissive of peptides with all carboxy-terminal amino acids except proline and glycine (Momburg et al. 1994), whereas mouse TAP is restricted to the transport only of peptides with hydrophobic carboxy termini (Heemels et al. 1993).

Recent studies has demonstrated a physical association between class I molecules and the TAP complex in the ER (Ortmann et al. 1994; Suh et al. 1994). This binding is thought to promote peptide capture by class I molecules before the peptides are exposed to the luminal environment. As a consequence of peptide binding, the class I molecules dissociate from TAP (Ortmann et al. 1994). The results of this study also showed that appropriate trimming and loading of the peptides into class I molecules and their presentation at the cell surface can occur in the absence of the TAP complex. Cytosolic peptides can additionally enter the ER in a TAP independent manner, via the normal SRP (signal recognition particle) dependent pathway (Forquet et al. 1993) and signal sequences of ER-targeted proteins are found to be presented by HLA-A2 molecules (Van Bleek and Nathenson, 1993). Recently, Elliot and co-workers (1995) have shown that in a TAP deficient cell line, large fragments of influenza nucleoprotein (NP) could be translocated into ER via a hydrophobic signal sequence. These large fragments were trimmed to a shorter peptide epitope of 9 residues for recognition by CTLs.

The typical view of class I processing and presentation is that proteasome degrade cytosolic proteins to 8 or 9 mer peptides which are then transported into the ER by ATP-dependent TAP transporters, prior to association with class I molecules. However, processed peptide ligands for class I molecules cannot be detected in the cytosol. Furthermore, in order to be selected by class I molecules, the entire pool of peptides would need to be transported into the ER. Rammensee and colleagues

have proposed an alternative model of class I processing which assumes an instructive role for class I molecules (Rammensee et al. 1993; Howard and Seelig, 1993). They suggest that proteins are first degraded into precursor peptides with appropriate carboxy termini for class I binding. These peptides would then be transported into the ER and bind with low affinity to fitting class I molecules via their carboxy-termini. The amino terminus would then be trimmed, allowing the peptide to snap into the groove and bind with high affinity. This model would require the transport of only a subset of available peptides and would fit with the current evidence for selectivity of the peptide transporters.

1.4.2 *Class II molecules*

The similar pattern of sequence polymorphism and conservation made possible an accurate prediction of the structure of the class II molecule from the class I structure, despite minimal sequence homology (Brown et al. 1988). Class II molecules consist of an α and a β chain, both encoded by closely linked MHC genes. The extracellular region of each subunit is divided into two domains α_1 - α_2 and β_1 - β_2 , each about 90 amino acids long. Class II molecules are mainly expressed on antigen presenting cells (APC) and B cells. In the class II MHC molecules, the peptide binding groove is formed by the interaction of the α_1 and β_1 domains, with the class II β_1 domain corresponding to the class I α_2 domain (Brown et al. 1993).

The structure of a class II molecule has been determined by x-ray crystallography of the class II HLA-DR1 molecule and is similar to the class I HLA structure and also to the earlier predicted class II structure (Brown et al. 1993). Class II molecules differ from class I primarily in the separation of the antigen binding domain between the two subunits, with one helix and four β strands contributed by each subunit.

Despite their overall similarity, differences between the structure of the class I and class II peptide binding grooves have important functional consequences. The most obvious is that the grooves of class I molecules are blocked at both ends, while those of class II molecules are open, allowing peptides to protrude. This is due to slight differences in the α helical regions of the class II antigen binding site (ABS). In class I molecules, the groove tapers at both ends and is then blocked completely by bulky amino-acid side chains, including Tyr 84 and Trp 167, which are conserved in virtually all class I MHC sequences (Saper et al. 1991). In the class II MHC molecule HLA-DR1, the blocking side chains are either replaced by smaller amino-acids or/ are repositioned by secondary structure changes concentrated at the ends of the cleft. Consequently, class II molecules bind longer peptides (12-24 residues), and specificity is confined to the conserved residues distributed throughout the binding site (Rudensky et al. 1991; Hunt et al. 1992). Another difference in comparison to class I structures is that DR1 crystallised as a dimer of the $\alpha\beta$ heterodimer i.e. a dimer of dimers. It has been suggested that this dimer of dimers may influence T cell signalling by affecting CD4 binding and TCR crosslinking (Ploegh and Benaroch, 1993; Brown et al. 1993).

1.4.2.1 *Assembly and intracellular transport of class II molecules*

Class II molecules typically assemble in the ER with a nonpolymorphic protein termed the invariant chain (Ii) (Claesson-Welsh and Peterson, 1985). The nine-chain complex formed is composed of three class II dimers and three Ii chains (Lamb and Cresswell, 1992). Various functions have been ascribed to Ii, and it is clear that it plays a central role in class II assembly and transport. The invariant chain directs the $\alpha\beta$ dimers to the class II loading compartment which appears to be in specialised vesicles characterised by the presence of class II molecules. The second function of

Ii is the prevention of premature peptide loading to the class II molecules. Avoidance of such binding is mediated by a short internal segment, the class II-associated invariant chain peptide (CLIP) invariant chain (Riberdy et al. 1992) which occludes or closes the class II ABS so that no peptide can bind. The delivery signal is located in the cytoplasmic tail of the invariant chain. Studies conducted on cell lines and mice deficient in invariant chain showed reduced assembly of class II α and β chains, inefficient transit to the cell surface of those class II dimers that do form, and reduced stability of class II molecules (Anderson and Miller, 1992; Viville et al. 1993). These results confirm the importance of Ii chain in class II assembly and transport. During the last stage of transit to or following entry into the major endosomal or lysosomal processing compartment, a combination of acidic pH and specific proteases progressively cleaves the COOH-terminal portions of Ii (Blum and Cresswell, 1988). A major role for Cathepsin E has been proposed for the degradation of invariant chain (Roche and Cresswell, 1991). Inhibition of the degradation of the Ii by Leupatin (and also lysosomotropic agents like NH_4Cl or Chloroquine) inhibit the transport of class II molecules to the cell surface. The rate of breakdown of the invariant chain determines the endosomal transit time of the class II molecules which varies from 1-3 hrs before they appear at the cell surface (Neefjes et al. 1990). The peptides loaded onto class II molecules can be derived not only from endocytosed proteins but also from proteins endogenous to the cells, especially membrane bound proteins which can co-localize in the class II binding compartment. At the cell surface, a small fraction of empty class II molecules can bind peptide. Only the newly synthesized pool of class II molecules, not the preexisting cell surface pool, associates with peptides derived from internalised antigen. This excludes a major functional role for the fractions of recycled class II molecules in presentation of unprocessed exogenous antigen (Davidson et al. 1991).

However, the role of recycled class II molecules in antigen presentation remains controversial. Recently, Pinet and co-workers (1995) have reported that recycled HLA-DR molecules were able to present immunodominant epitopes from the haemagglutinin protein of influenza virus. It was also suggested that the compartment for loading of peptide onto recycled class II molecules is probably distinct from the one where newly synthesised class II molecules are loaded.

1.4.2.2 Class II processing

Recognition of antigen by MHC class II restricted T cells requires processing of the native antigen into peptides (Unanue and Allen, 1987). Class II molecules obtain their peptides predominantly from extracellular sources by endocytosis, with subsequent degradation in acidified endosomes or lysosomes. The precise location and nature of the acidic peptide loading compartment has, until very recently, been ill defined. The prevailing view has been that class II/Ii complexes meet the unprocessed antigen in an endosomal compartment, and together proceed along a proteolytic pathway so that the unoccupied class II molecules are produced simultaneously with antigenic peptides. Several workers have recently reported the identification of a unique subcellular compartment, the CPL (the compartment for the peptide loading), in which class II loading occurs (Amigorena et al. 1994; Tulp et al. 1994; West et al. 1994). The CPL have no detectable endosomal or lysosomal markers and have distinguishable subcellular fractionation behaviour (Schmid and Jackson, 1994). Most class II molecules in the CPL are not associated with Ii. It has been suggested that the CPL undergoes a maturation process whereby Ii is degraded, class II dimers assume an intermediate conformation and peptide loading occurs subsequently.

1.5 MHC ligands and peptide motifs

Huge advances have been made in recent years in the analysis of peptides naturally presented by class I MHC molecules. Naturally processed peptides associated with class I and class II MHC molecules are usually isolated from immunoaffinity purified material, followed by size fractionation to remove the MHC proteins themselves (Buus et al. 1988; Van Bleek and Nathenson, 1990). However, the binding of some antibodies to MHC molecules can be significantly altered by the structure of the associated peptides (Bluestone et al. 1992), and isolation of some peptide-MHC complexes may therefore depend upon the antibody used. Peptides eluted from purified class I molecules can be separated by HPLC and their sequences analysed by Edman degradation and /or mass spectrometry. A special application of Edman degradation is pool sequencing, that is, sequencing of the complex mixture of peptides eluted from a given MHC species (Falk et al. 1991). The most sensitive means of detecting the isolated peptides is the T-cell assay, which can detect peptides in sub-picomolar range, in cytotoxic T cells assays (Rotzschke et al. 1990). The major shortcoming of the T-cell assay in peptide detection is that it does not give sequence information. Such studies have revealed that each class I allelic molecule has its own peptide specificity or peptide motif (Van Bleek and Nathenson, 1993; Hunt et al. 1992; Falk et al. 1991). Pool sequencing of MHC class I ligands led to the discovery of allele-specific motifs, and a large number of such motifs have now been determined (e.g., H-2L^d, HLA-B7, HLA-B*5102 and HLA-B*5103), (Rammensee et al. 1995). Recently, the peptide motifs for two bovine class I alleles BoLA-A18 and BoLA-A11 has been reported (Bamford et al. 1995; Hedge et al. 1995). Peptides bound to class I molecules are restricted to 8 or 9 residues in length.

The peptides bound to the same class I molecules were found to share sequence motifs which complement the binding pockets in the class I molecule in question. The specific interaction between a peptide and class I MHC molecule is mediated through residues on the peptide, whose side chains protrude into complementary pockets of the class I groove (Guo et al. 1993). The amino and carboxy-terminal ends of the peptide bind with conserved residues in two pockets located at opposite ends of the binding site, termed A and F, respectively (Madden et al. 1992). Although all class I molecules analyzed to date appear to bind peptides in a common orientation, polymorphic residues located in pocket F and four more centrally located pockets (B, C, D and E) influence the specificity of peptide binding or bound peptide conformation. Each peptide motif has anchor positions and these also vary depending upon the class I molecule involved. Without exception, one anchor is found at the carboxyl terminus of the peptide. These carboxyl terminal anchors are generally hydrophobic or charged. The carboxy-terminal peptide residue is an important determinant for binding of peptides associated with all class I MHC molecules. The peptides associated with most class I molecules also show motif residues at position 2 (P2) relative to the amino terminus, which interacts with the B pocket of the binding cleft. For some class I molecules, (e.g. HLA-C, Falk et al. 1993a), a second anchor appears to be replaced by two or three auxiliary anchors reflecting either MHC pockets with degenerate specificity, or the alternative use one of at least one of two or three specific anchors. For most cases the determined (Madden et al. 1993), or predicted pocket structure allows a satisfying explanation for pocket specificity. For example, amino acid residues 77 and 116 of the class I heavy chain contribute to a pocket accommodating the carboxyl terminal anchor side chains of peptide (Madden et al. 1992). If both of these residues are negatively charged, as in HLA-A31, for example, the corresponding anchor is a positively side

chain (Falk et al. 1994). If only one of the residues is negatively charged e.g. as in HLA-A1, a positively charged carboxyl-anchor side chain of the peptide P-C is no longer allowed. Similar considerations can be made for the P2-accommodating pocket, so that attempt can be made to predict MHC motifs almost entirely on theoretical grounds (Thorpe and Travers, 1994). However, it has been suggested that such interpretations should be made with caution, especially when the MHC molecule in question has not yet been crystallized, and the pocket structure has only been inferred by sequence homology. In addition to anchor residues, several, if not all, of the other peptide residues can make contact within the MHC groove but these interactions differ with different peptides.

Information on allele-specific motifs can be used to identify T-cell epitopes within proteins of known sequence and detailed analysis of peptide/MHC interactions. The successful identification of peptides presented by MHC molecules that originate from viruses or pathogenic agents will certainly lead to the identification of better candidate antigens for vaccines, but also to development of new strategies for autoimmune disease and tumor immunology.

An elegant approach for studying the peptide specificity of class II molecules has been developed by Hammer and co-workers (1994). A peptide library was expressed by bacteriophages. From the peptide expressing phages only those phages were selected which bound to a given class II molecule. The peptide sequences expressed by the selected phages were then determined. Motifs obtained with this method for HLA-DR1 has been established and were in accord with the known crystal structure of the HLA-DR1.

1.6 Molecular cloning of class I genes in man and mouse

The pioneering works of Steinmetz et al. (1982a, 1982b) and Weiss et al. (1984) provided the first insights into the molecular organisation of two murine MHC haplotypes. These investigators carried out large scale chromosome walking in the genomes of BALB/c (H2^d) and C57Bl/10 (H2^b) and linked several clusters of structurally related genes within H-2 complex. The molecular analyses of the human MHC (HLA) began soon after the isolation of cDNA clones for HLA-B7 toward the end of 1980 (Ploegh et al. 1980; Sood et al. 1981). Once isolated, these cDNA clones were used to build a picture of class I loci in humans and mice by the dual approach of southern blotting and genomic cloning. The complete mapping of the class I region in man and mouse reveals that numerous genes are present, and that, remarkably, there appears to be some conservation of genes between the species. The human MHC map (Campbell and Trowsdale, 1993; Fig 1.2) is built on a combination of mapping by pulse field gel electrophoresis (PFGE: Hardy et al. 1986) and analysis of overlapping genomic clones in cosmid, bacteriophage λ and yeast artificial chromosome (YAC) vectors (Newell et al. 1994).

1.6.1 *Class I gene structure*

Southern blotting studies in mice highlighted the large numbers of class-I-hybridising sequences in the genome; the majority of these which mapped outside the class I K and D regions to the Qa and Tla regions. One of the first gene sequences to be reported mapped to the Qa region in BALB/c mice; H-2Q7^d had eight exons but contained a stop codon which gave a truncated gene product (Steinmetz et al. 1981b). The intron/exon structure of this clone mirrored the domain assignments of class I protein structure, with exons corresponding to each of the three external domains and the transmembrane and cytoplasmic regions. Other gene sequence

Fig. 1.2 The organisation of the human MHC complex.

The map of human MHC including the genes encoding for class I, class II, class II, transporter and LMP molecules.

included H-2L^d (Moore et al. 1982) which was also found to encode eight exons. The fine structure of the sequenced clones showed the eight exon format was very well conserved in the class Ia and the class Ib genes in mouse (Steinmetz et al. 1981b; Evans et al. 1982a) and man (Malissen et al. 1982; Geraghty et al. 1987) and can be dissected as follows:

5'-untranslated (5'-ut) :- This region normally varies from 20-30bp in the area between the TATA Box and translation start.

Exon 1 :- The first exon encodes the leader peptide in the immature protein and varies from 21 (Lalanne et al. 1982) to 24 residues in man (Srivastava et al. 1985) although exceptions include HLA-A3 which has a leader peptide of 29 residues (Strachan et al. 1984).

Exons 2, 3 and 4 :- These encode the three extracellular domains of the protein which are 90, 92 and 92 amino acids long respectively. Exons 2 and 3 encode the polymorphic antigen binding site, exon 4 is relatively conserved and binds with β_2m . All these domains show structural similarities to immunoglobulin constant regions. The structure of these domains appear to be very well conserved across the species.

Exon 5 :- This encodes the 40 amino acid transmembrane region of the protein. This domain is the most variable in length in class I genes from different loci within species, and has been suggested to contain locus specific substitutions. In human, HLA-C products have one more amino acid than HLA-A and HLA-B. In the bovine MHC, the different sequences isolated have also been shown to vary in the length of the TM region. In horses, five different sizes of TM have been reported (Ellis et al. 1995).

Exon 6, 7 and 8 :- These encode the cytoplasmic domain and the exact role of this domain is not clear. Influenza-infected cells expressing a transfected H-2L^d

molecule lacking a cytoplasmic domain were recognised by cytotoxic T-lymphocytes, but similar vesicular stomatitis virus infected cells were recognised less easily without the cytoplasmic domain (Murre et al. 1984). A serine in this domain is highly conserved, and may be phosphorylated, which suggests a function, for this conserved sequence. A role for this domain in intracellular transport (Zuniga and Hood, 1986) and endocytosis (Capps et al. 1989) has also been suggested. Exon 6 is normally 11 amino acids long in class I genes. Exon 7 can vary in length from 13 a.a. (H-2L^d, H-2K^d) to 16 a.a. (HLA-Cw3) and exon 8 also varies in length from 1 a.a. in H-2L^d and HLA-A genes, up to 10 a.a in H-2K^d. In all of the HLA-B locus genes sequenced so far, a termination codon is found at the end of the exon 7, so that B locus genes lack the residues encoded by exon 8 (Gussow et al. 1987).

3' untranslated region :- It can vary in class I genes from approximately 300bp up to 900bp for Tla^c. This region lacks the structural constraints of coding regions, and has retained less nucleotide similarity between the different class I genes.

Most of the variation in length of the molecule is generated by exons 6, 7 and 8. This includes variation in length by alternative splicing of exons (Steinmetz, 1981a). Several instances of class I alternative splicing have been described: for example in the mouse H-2K^b, H-2K^q, H-2K^k and H-2L^d (Lew et al. 1987; Vogel et al. 1989), in human HLA-A24, HLA-A2 (Krangel 1986), and possibly HLA-C (Mizuno et al. 1989), in sheep and bovine class I genes (Grossberger et al. 1990; Ellis et al. 1992). Most of these examples involve exons 7 and/or 8, and lead to truncated molecules. Many non-classical class I genes also demonstrate alternative splicing of exons 5-8

(Lew et al. 1987; Ulker et al. 1990). Transfection studies with alternatively spliced class I molecules (Zuniga and Hood, 1986) indicate that structural variations in the cytoplasmic domain can lead to the use of distinct pathways of intracellular processing and can effect the way the molecules interact with the cytoskeleton.

Variations in the length of class I molecules can also be caused by in-frame termination codons. This is particularly frequent in mouse Qa genes which all appear to have a 8 exons, but have a termination codon in the fifth exon giving rise to truncated proteins (Steinmetz et al. 1981b; Mellor et al. 1984; Robinson et al. 1988). Although the structure of class Ib Qa genes is relatively similar to the classical class I genes, this is not the case for the Ta genes. In these genes the first five exons follow the same format as the classical class I genes, but exon 6 encodes the entire cytoplasmic region of Ta^b, which is two amino acids longer than H-2L^d (Fisher et al. 1985).

1.6.2 *Class I genes in the mouse major histocompatibility complex*

The Murine major histocompatibility complex H-2 encodes the classical transplantation antigens K, D and L which are related to A, B and C in man. The genetic nomenclature of the H-2 complex is complicated and confusing. A particular gene is designated by a capital letter (eg. K, D or L) for the locus and each locus has alleles designated by a small letter superscripts in complex combination with H-2 symbol (eg K^a, K^b, D^a). The availability of inbred strains of mice congenic for MHC haplotypes (eg. BALB/c and C57BL line which are classified as H-2^d and H-2^b haplotypes respectively) has facilitated extensive genetic analysis of this system. Adjacent to the loci encoding these molecules on chromosome 17 is the Ta region, which contains genes encoding the Q and T lymphoid differentiation antigens. These

antigens, like class I antigens, are integral membrane proteins with molecular weights of 40-45,000 D (Soloski et al. 1981) and are expressed on the cell surface in association with β_2m (Coligan et al. 1981), but Q and Ta are relatively non-polymorphic (Flaherty et al. 1980). Cell mediated lymphocytosis (CML) can be directed against Q antigens but is not MHC restricted (Kastner et al. 1979a), and Q restricted CTLs cannot be generated (Kastner et al. 1979b).

1.6.3 *Molecular cloning of class I genes in mouse*

Steinmetz et al. (1982b) identified 36 distinct class I hybridising sequences from BALB/C mouse (H-2^d) and subsequent mapping placed 31 of these in the Q and Ta regions (Winoto et al. 1983). This was done using congenic strains of mice that had undergone recombination in the MHC. The strategy used was to digest cosmid clones with frequently cutting enzymes to construct an overlapping map of the cosmid so that the genes could be ordered into clusters. These cosmid clusters were mapped to the genetically defined regions of the H-2 complex by restriction fragment length polymorphism (RFLP). The RFLP mapping strategy relied on the isolation of probes from the cosmids which detect unique or low numbers of genomic DNA restriction fragments. These probes were used against DNA from congenic or recombinant strains of mice to detect restriction site polymorphisms. Essentially three types of polymorphism can be detected 1) Change in fragment size (mutation), 2) Loss of the fragment (deletion), 3) an increase in the number of fragments (duplication). These polymorphisms can be correlated with serological polymorphisms, allowing the mapping of a particular fragment to one of the four class I regions characterised. The techniques of Southern blotting and genomic cloning highlighted several new findings. The first was that class I genes could vary in number in different haplotypes of mice. There is considerable divergence in the

number of class I genes located in the H-2D/H-2L region of BALB/C (H-2^d) and B10 (H-2^b) mice (Weiss et al. 1984; Stephan et al. 1986). The D region of BALB/C mice contains 5 genes (H-2D^d, D2^d, D3^d, D4^d, and H-L^d). This contrasts with the single gene characterised in the D region of B10 mice (Weiss et al. 1984). The L^d gene is most distal in this region and is separated by 60kb of DNA from the most proximal gene of the Q region in Q1. However, the organisation of the H-2K region is similar in BALB/c and B10 mice. Both these strains have two genes in H-2K region, K and K2. Only the K gene appears to be expressed.

The second major finding was that the main source of variation in the numbers of class I hybridising bands seen in genomic hybridisation studies was in the Q and Ta genes. In contrast to the polymorphic K and D antigens, the Q and Ta antigens lacked functional polymorphism as detected by serology, but showed a higher degree of numerical polymorphism detected at the DNA level. This is discussed in more detail in section 1.9.1.

1.6.4 *Molecular cloning of class I genes in man*

In order to find different class I loci Koller and co-workers (1989) derived locus-specific probes from class I genomic clones detecting restriction fragment length polymorphisms (RFLP) on genomic DNAs. These probes were used to analyse a collection of mutant human lymphoblastoid cell lines derived from B-LCL 721. By these methods 17 class I genes were mapped in the MHC region; 11 of these were pseudogenes, three corresponded to HLA-A, -B and -C, and the remaining clones corresponded to HLA-E (Koller et al. 1988), HLA-G (Geraghty et al. 1987) and HLA-F (Shimizu et al. 1988). The class I gene family located towards the telomeric end of chromosome 6 and a rough estimate of gene density was one gene every 20kb

(Geraghty et al. 1993; Wei et al. 1993). Lamm and Olaisen (1985) showed, by linkage analysis with serological reagents, that HLA -A, -B and -C loci were close together. HLA-B was most centromeric at 0.1cM from the HLA-A locus, while the estimated genetic distance between HLA-A and HLA-C was 0.7cM. Carroll and co-workers (1987), who analysed PFG patterns after DNA digestion with several rare-cutter restriction enzymes, provided evidence of the physical distance of 250kb separating HLA-B and HLA-C. In the case of the HLA-B and HLA-C genes, locus specific probes, derived from the 3' region of both genes were used to detect two distinct fragments in *Mlu* I digested DNAs. By the use of linking probes, the HLA-B and HLA-C genes were found to be in the same transcriptional orientation on the chromosome with the 5' end centromeric to the 3'-end. Other probes derived from class I genes such as p12.4 or cda12 were used to physically map the area surrounding the HLA-A locus: p12.4 lies within 200kb (Pontarotti et al. 1988) and cda12 within 50kb (Ragoussis et al. 1989) of the HLA-A locus. These data are consistent with the earlier observations that the majority of the 20 unidentified class I-like genes clustered around the HLA-A gene, while the HLA-B and HLA-C genes are isolated (Chimini et al. 1988). The overall length of the class I region according to this data is at least 1800kb and the whole region contains approximately 20 class I related sequences. It seems highly probable that additional genes are present in this region. Geraghty and co-workers (1992) isolated a series of overlapping YAC clones in order to clone and map the entire HLA class I region. Using this approach they identified all the possible HLA class I genes in this area.

A more recent strategy has been to isolate cDNA clones of the entire coding region from individual loci by using polymerase chain reaction amplification with oligonucleotide primers that hybridize with sequences in the 5' and 3' untranslated

regions (Ennis et al. 1990). The 3' flanking sequence contains a pattern of nucleotide substitutions that are characteristic of the individual loci and could be used to design 3' primers giving preferential amplification of either HLA-A, B or C alleles in human (Zemmour et al. 1992).

The HLA-A, HLA-B, and HLA-C antigens are expressed on the surface of almost all nucleated cells (Ploegh et al. 1981). In addition to functioning as strong transplantation antigens, these molecules also play a crucial role in the presentation of viral and tumor antigens to cytotoxic T lymphocytes (McMichael et al. 1977; Wallace et al. 1982). Their assignment to specific alleles was made possible by expression studies after transfection in mouse Ltk- cells (Lemonnier et al. 1982, 1983). To date, complete coding region sequences have been determined for 43 HLA-A, 89 HLA-B and 26 HLA-C alleles (Parham et al. 1995). Unlike tissue typing for HLA-A and HLA-B, a large percentage of individuals cannot be typed for at least one HLA-C allele using human alloantisera, perhaps because of poor cell surface expression (Takiguchi et al. 1989; Hajek-Rosenmayr et al. 1989). Gussow and co-workers (1987) observed poor cell surface expression in mouse L cells transfected with cloned genomic Cw1 and Cw2, genes, of about 10% of the levels of HLA-A and HLA-B. In this regard, HLA-C is similar to the H-2L^d molecule (Dower and Segal, 1985) which is expressed on the cell surface at levels three or four times lower than D^d or K^d (Beck et al. 1986). This poor cell surface expression is contributed to by inefficient assembly with β_2m and a slower rate of transport to the cell surface (Neefjes and Ploegh, 1988), and so the importance of these molecules in antigen presentation was considered negligible. The question of functional significance was addressed by Dill and co-workers (1988) using HLA-Cw3 transgenic mice. These transgenics were able to produce HLA-Cw3-restricted CTL against

influenza and Sendai virus. Thus, one HLA-C allele has been shown to be functional as an antigen presenting molecule.

1.7 Identifying functionally important regions in class I genes

Recombinant DNA technology has been used to dissect out the functionally important exons of class I genes by exchanging homologous exons between different class I molecules whilst retaining all the information necessary for correct expression. Studies utilizing HLA hybrid genes have revealed the association of serological epitopes with first two extracellular domains α_1 and α_2 (Jordan et al. 1983). Hybrid murine class I genes were constructed between the following gene pairs: H-2D^d, H-2L^d (Evans et al. 1982b; Murre et al. 1984); and H-2K^d, H-2K^b (Arnold et al. 1984). These hybrid genes were reconstructed by joining the exons encoding the leader peptide, α_1 and α_2 regions from one class I gene to the exons encoding α_3 , TM and cytoplasmic regions from a second class I gene. Functional analyses performed on the chimeric molecules showed that the majority of the anti-H-2 monoclonal antibodies recognized determinants that are encoded by α_1 and α_2 regions. Thus, the antigenic specificity of a hybrid gene correlated with the antigenic specificity of the product of one of the donor genes (encoding α_1 and α_2) rather than the other donor gene (encoding α_3 , TM and the cytoplasmic regions). While a small number of monoclonal antibodies did recognize determinants encoded by the α_3 domain, alloreactive and self-restricted CTL predominantly detected polymorphic epitopes within α_1 and/ or α_2 but not in α_3 (Allen et al. 1984; Ozato et al. 1983). To test if monoclonal antibody or CTL recognition sites reside in a single polymorphic domain, the leader peptide and the α_1 -encoding exon from H-2D^d was joined to the α_2 , α_3 , TM and cytoplasmic encoding exons from H-2L^d and vice versa (Ozato et al. 1985). Serological analyses of these constructs indicated that some antibodies recognized

determinants residing in the α_1 and α_2 independently domains while others detected determinants resulting from the interaction of domains α_1 and α_2 . These observations suggested that there were strong interactions between the domains, so that even where a polymorphic determinant only involves a stretch of amino acids from one domain, its conformation is dependent on other domains of the molecule. Most allospecific and virus-specific CTL were shown to behave in a similar fashion. Therefore, it was suggested that the polymorphic epitopes recognized by T cells are controlled by independent domains.

1.7.1 *Site-directed mutagenesis for mapping the functional important areas of class I molecule*

Class I molecules could also be altered *in vitro* by site-specific mutagenesis, allowing even more detailed examination of the functionally important areas. Analysis of such mutant H-L^d gene products lacking the disulphide bridge of the α_2 domain as a result of replacing the Cys 101 with Ser, resulted in near-total destruction of the epitope recognised by allo-specific CTLs (Shiroishi et al. 1984). Diminished reactivity with anti-L^d monoclonal antibodies specific for the α_2 domain was observed, but antibody reactivity was retained for the α_1 and α_3 domains on the substituted gene product. It was suggested that the disulphide bridge controls only intradomain structural features.

A more detailed analysis of *in vitro* generated mutants suggested that the α_3 domain also has a crucial role in the correct folding of these molecules. Site directed mutagenesis of. Glu 227 to Lys lost CTL recognition of H-2D^d (Potter et al. 1987). It appears that the altered residue is recognised by CD8-independent T cells but not by CD8-dependent T cells (Potter et al. 1989). CD8 is an accessory molecule which

is a phenotypic marker of CTL and which interacts with class I molecules during antigen presentation.

The conclusions reached from studies on exon shuffling and mutagenesis suggest that the interaction between the first two domains is important in generating conformational sites detected by allogenic and restricted CTL. In summary; a) small changes in the first or second domains can cause significant alterations in recognition by antibodies or CTL; b) CTL recognise conformational determinants produced by interactions of residues located at sites in the first, second and third domains of class I heavy chain polypeptides.

1.8 MHC polymorphism

In most species studied, polymorphism in the MHC loci is extensive and has been maintained through time (Klein, 1987). This feature of MHC molecules is central to their role in presenting a diverse range of peptides, thereby providing protection (at the population level) against evolving pathogens. Sequence polymorphism in MHC molecules results in differences in the detailed shape and charge distribution in the antigen binding site, but does not significantly alter the overall structure, which appears to be well conserved between mammalian species. The selective advantage of polymorphism at the class I loci provides the basis for presentation of a wide range of pathogen peptides, but the increase in the range of antigens recognised may be offset by a reduction in the T cell repertoire (Matzinger et al. 1984). This may account for the small number of functional class I loci (usually one, two or three) seen in all species studied (Robinson and Kindt, 1989), although there may be additional genes coding for less ubiquitously expressed class I molecules.

Many questions remain about the origins of MHC polymorphism and its functional significance. Gene conversion or non-homologous interlocus recombination, where a unidirectional exchange of information occurs (Bregg, 1983), has been suggested as a mechanism for the generation of polymorphism in the MHC. For example, a mutant allele H-2K^{bm1} of the H-2K^b gene was cloned by Weiss et al. (1983a), which differed in three codons and was shown to be a result of gene conversion. The altered residues in H-2K^{bm1} were identical to those of H-2L^d. They proposed that H-2L^d was the donor for the gene conversion event. Unfortunately, this gene was absent from the haplotype in which the conversion event was proposed to have happened. However, Mellor et al. (1983) provided evidence for Q10 as the potential donor gene for the H-2K^b to H-2K^{bm1} gene conversion in C57BL/10. Weiss et al. (1983b) also showed that several of the differences in nucleotide sequence between the H-2K^b and the H-2K^d alleles could be explained by a similar gene conversion event.

More evidence for gene conversion came from the generation of two HLA-B27 subtypes of human class I genes (Seemann et al. 1986). Comparison of class I HLA allelic sequences gives a quite a different picture from that seen with class I H-2 alleles, in that gene conversion between different loci has made a relatively minor contribution to polymorphism (Parham et al. 1989). Instead, it appears that intragenic or allelic conversion is the main mechanism of generating HLA polymorphism. Holmes and Parham (1985) observed such recombination in the HLA-Aw69 allele, which they suggested was due to *in vivo* exon shuffling between the α_1 domain of the HLA-Aw68 and the α_3 domain of HLA-A2. It was also observed that positions of high variability were directly involved in peptide-binding as defined by the structure of HLA-A2 (Bjorkman et al. 1987a).

Another mechanism suggested for generation of the polymorphism has been gene duplication. The variation in the number of class I genes among various mouse strains has been suggested to be due to gene duplication. Weiss and co-workers (1984) reported that Q region genes mainly evolved by genes duplication. By the use of restriction mapping and hybridization they showed that the Q5, Q7 and Q9 genes were similar to each other, and likewise that the Q6, Q8 and Q10 genes were closely related. They suggested that each of these three genes arose as a result of gene duplication. In addition, a great degree of similarity between the two genes in the H-2K region and the Q6-Q7 gene pair was also seen. These results suggested that the H-2K region was generated by translocation of the gene pair from the Q region or vice versa.

In man, HLA-A and HLA-AR are two closely linked genes which have been suggested to have arisen as a result of gene duplication of an ancestral gene. Although HLA-A is a functional class I gene, but all six alleles of the HLA-AR locus are pseudogenes due to substitution by phenylalanine of the cysteine at position 164 which forms an essential disulphide bond (Zemmour et al. 1990). In contrast to the HLA genes, in H-2 many interallelic differences are greater than interlocus differences, for example in some haplotypes, the class I K and D molecules of the same haplotype differ by fewer amino acids than do different allelic forms of the K products.

Hughes and Nei (1988) proposed that polymorphism is due to over-dominant selection (heterozygote advantage). They compared the rate of nonsynonymous substitution in the antigen binding site with that of the remaining amino acids in the first two domains. They found that the rate of nonsynonymous substitution was

much higher in the antigen binding site, while in the rest of the domain the reverse was true. Therefore the amino acid differences between class I alleles are localised to the functionally important areas of the molecule, and this probably accounts for the large number of alleles in the population via heterozygote advantage. The possession of two different alleles at a locus would have the advantage of allowing a response to more pathogens, so that heterozygotes would have a higher fitness than homozygotes.

An elegant explanation for MHC polymorphism has been provided by the trans-species hypothesis of (Klein, 1987; Arden and Klein, 1982). This theory assumes that ancestral forms of the major alleles were present before speciation. When a new species evolved, the whole set of alleles would be passed on when there are no drastic environmental changes. Additional polymorphisms would result only from random mutations. A truly new polymorphism would only be generated by drastic changes in the mode of life (Klein and Figueroa, 1986; Lawlor et al. 1988; Mayer et al. 1988). Evidence for this theory is seen in the studies of Mayer et al. (1988), who compared class I cDNA clones of chimpanzee with class I genes of man, and found 97.8% similarity between chLA-A108 and HLA-A11. This very high degree of similarity between species suggests that this class I allele originally arose in an ancestor of these two species.

1.9 Non-classical class I genes

In addition to the MHC genes, which are involved in the initiation of specific immune responses, there is a group of MHC genes whose functional status is uncertain. The two groups are often referred to as classical and non-classical MHC genes respectively. The term 'non-classical genes' is usually applied to the class I

loci, but in fact some of the families of class II loci (e.g. the mammalian DM system) also fit the description. Both human and mouse MHC regions contain a substantial number of non-classical (class Ib) class I genes. It was speculated that many non-classical class I genes were produced by duplication of classical class I genes and that the majority became non-functional by accumulating deleterious mutations (Hughes and Nei, 1989; Klein and Figueroa, 1986). The mouse MHC class Ib family consists of at least 30-40 genes encoded in the H-2Q, T and M regions whereas HLA-E, F, G, H, I and J regions have been identified for the human non-classical antigens (Shawar et al. 1994). Genes homologous to murine Q and T region genes have additionally been identified in the rat (Kirisits et al. 1994; Rothermel et al. 1993). These non-classical class I molecules are characterised by limited polymorphism, restricted tissue distribution, poor surface expression and short cytoplasmic domains (Stroyonowski et al. 1990; Flaherty et al. 1990).

1.9.1 *Non-classical class I genes in mouse*

The Q and M regions in the mouse each contain about 10 class I genes, and the T region has twice as many. The Qa-2 genes are expressed in a wide variety of tissues (reviewed by Morse et al. 1991). The Q2, Q5 and Q10 gene products have a more tissue-specific expression pattern. The Q4, Qa-2 and Q10 gene products can be detected as secreted molecules. The Q5 gene in H2^b haplotype has acquired a premature termination codon in exon 3 while the corresponding gene in H2^k strain is intact (Jaulin et al. 1985; Robinson et al. 1988). It has recently been reported that two forms of soluble Qa-2 exist- one form corresponds to the translational product of a truncated transcript, while the second form of Qa-2 is attached to the cell surface via a GPI (glycophosphatidylinositol) linkage and has been implicated in transmembrane signalling (Stroynowski et al. 1990). The Q6 and Q7 gene products

have been suggested to play a role in T cell restriction (Mann and Forman, 1988).

The T region gene products are predominantly expressed on normal thymocytes and thymic leukaemic cells. Earlier it was thought that T genes were non-functional (Rogers et al. 1986). However, various workers have recently reported the expression of T region gene products in the intestinal epithelium where they have been suggested to function as restriction elements for $\gamma\delta$ T cells (Wu et al. 1991; Hershberg et al. 1990; Eghtestady et al. 1992). Furthermore, cell surface expression of a transfected Q α -1 molecule was stabilised following heat shock and by addition of a tryptic mycobacterial digest (Imani and Soloski, 1990), suggesting a role in peptide presentation.

The most thoroughly studied non-classical class I genes are the M region genes of the mouse MHC (reviewed by Fischer Lindahl et al. 1991). This region of the mouse MHC is presently known to contain 8 genes (Wang et al. 1993). H-2M3 is the best understood example of a non-classical class I presentation element. It has been found that H-2M3 molecules bind and present peptides with a unique structural component of prokaryotic proteins, N-formyl methionine (Shawar et al. 1990). The presentation of such peptides is TAP- dependent and the availability of endogenous peptides carrying the N-formyl group limits surface display of H-2M3 on mammalian cells (Vyas et al. 1994) suggesting that it can be expressed at high levels only during infection by prokaryotic organisms. It has been subsequently been demonstrated that H-2M3 can indeed present bacterial peptides to CTL during *Listeria monocytogenes* infection (Pamer et al. 1992; Kurlander et al. 1992) providing a formal proof of the involvement of this class Ib antigen in the immune response against an intracellular pathogen. Wang and colleagues have proposed that

the HMT molecule may be considered as a neoclassical class I molecule, since its developmental expression and pattern of cytokine induction parallel that observed for classical class I molecules (Wang and Fisher Lindahl, 1993).

1.9.2 *Non-classical class I genes in man*

The human MHC was originally thought to contain only 15 class Ib genes or gene fragments. The four non-classical HLA class I loci include HLA12.4 (HLA-H, Chorney et al. 1990), RS5 (HLA-E, Orr, 1989), HLA6.0 (HLA-G , Koller et al. 1989) and HLA5.4 (HLA-F, Geraghty et al. 1987, 1990).

The lack of surface expression of HLA-12.4 has been attributed to the substitution of cysteine 164 by phenylalanine in the α_2 domain of this molecule, disrupting the correct folding of the class I protein. Two class I sequences RS5 and HLA6.2 have been assigned to the HLA-E locus (Koller et al. 1988). Both of these sequences have more than 97 % homology with each other and have unique features that distinguish HLA-E loci from all other loci reported. The sequence of the TATA box (TCTAA) in the RS5 gene has undergone a A→C transition at the second nucleotide (Srivastava et al. 1985) and thus resembles the TATAA sequence found in the class I genes (Hedley et al. 1989). This gene has also acquired a frameshift alteration in the seventh exon, and thus encodes a shorter cytoplasmic tail of 29 amino acids. The HLA-E transcript appears to be ubiquitously expressed at low levels in most adult tissues and in placenta (Koller et al. 1988).

The distinguishing feature of the HLA-G locus is the presence of termination codon at the start of the exon 6 (Koller et al. 1989). Thus, HLA-G products have only six amino acids in their cytoplasmic tail as opposed to 29 to 32 amino acids in other

human class I proteins. The HLA-F non-classical class I gene has a defective splice junction. HLA-F related transcripts have been detected in B cell lines, resting T cells and in skin cells. HLA-G expression has been found only in foetal tissue. Several studies have reported its expression in placenta and extraplacental membranes (Ellis et al. 1990; Kovats et al. 1990) and is assumed to be involved in materno-fetal interactions. Shukla and co-workers (1990) also reported its presence in the foetal eye and thymus.

Although speculations have been made for a possible role of non-classical class I molecules, there is no strong evidence for their proper function. Klein and colleagues have long held the view that the non-classical class I genes constitute an evolutionary junkyard (Klein et al. 1991). They consider that these genes have no functional significance and that they represent relics of genes once used in antigen presentation or the products of unsuccessful attempts at producing new genes. It has been proposed that these genes may provide a source of donor sequences for the generation of sequence variation in classical class I genes (Lawlor et al. 1990). Others have suggested that the non- classical class I genes may function in a manner analogous to the classical class I molecules (Strominger, 1989; Srivastava and Lambert, 1991).

1.10 Molecular cloning of class II genes in man and mouse

The class II products are encoded in the HLA-D region of the human MHC and consist of 14-16 genes (Hardy et al. 1986). They were initially defined in mixed lymphocyte cultures (MLC) using homozygous typing cells. By use of serological techniques it soon became clear that there were several class II loci (HLA-DR, HLA-DQ, HLA-DP). The gene products of the class II family present peptides of

extracellular pathogens to T cell helper/inducer subsets. They are expressed on macrophages and B lymphocytes and other professional antigen presenting cells. Three families of class II genes have been identified in the HLA system, HLA-DP, DQ and DR (Trowsdale et al. 1984; Okada et al. 1985), each consisting of a distinct α and β chain. The number of *DRB* genes appears to vary between human haplotypes (Bell et al. 1987; Bohme et al. 1985). Cosmid cloning from the three *DRB* gene DR3 haplotype and the four *DRB* gene DR4 haplotype (Rollini et al. 1985; Spies et al. 1985) confirmed the variations in *DRB* gene number. The existence of DP, DQ, and DR antigens might serve to diversify the range of immune responses that each individual can mount. However, other class II loci might exist. The class II region also contains a group of genes (RING3, 6, 7, 12, 4, 9, 10 and 11; Hanson et al. 1991; Trowsdale et al. 1991; Kelly et al. 1991a) for which no clear function was initially seen. A role also remains to be found for the *DNA* and *DOB* products, equivalent to the non-polymorphic Oa and Ob genes in mouse (Tonnellet et al. 1985; Karlsson et al. 1992). RING 3 is the only one of these genes remaining with unknown function (Beck et al. 1992). RINGs 10 and 12 (LMP-2 and LMP-7 respectively) are thought to encode components of the antigen processing pathway for class I molecules (Kelly et al. 1991b). RINGs 4 and 11 (TAP1 and TAP2 respectively) are thought to encode the transporter molecules involved in translocating antigenic peptides into the ER lumen in order to be coupled to class I molecules (Kelly et al. 1992). In recent nomenclature for HLA genes (Bodmer et al. 1994), RINGs 6 and 7 have been assigned to alleles of DM genes. The DM genes have been suggested for the correct loading of peptide onto class II molecules (Fling et al. 1994; Morris et al. 1994; Wolf and Ploegh, 1995).

The H-2I region, on the other hand, has two defined gene families I-A and I-E. The

I-A molecules are considered to be homologues of the molecules encoded by the HLA-DQ genes, and I-E are homologues of HLA-DR (Widera and Flavell, 1985). The H-2 class II region differs from the human primarily by the absence of genes equivalent to the HLA-DP genes. There also do not appear to be homologues of DNA, DQ2 or DQB2 genes or the human pseudogenes. No variation in number of the class II genes has been reported between different haplotypes of mouse.

The majority of genes identified in the human MHC to date are found in the class III region, a stretch of 1000 kilobase (kb) pairs flanked by the class II region on the centromeric side and the class I region on telomeric side. The class III region contains a range of tightly linked genes, some of which may be immune system components such as the C2, C4, Bf and TNF genes. (Campbell and Trowsdale, 1993). The class III region genes are neither structurally nor functionally related to class I or class II genes, and it has been suggested (Klein, 1986), that the class III region is not a part of the MHC, but resides on the same chromosome by coincidence.

1.11 MHC of other species

Apart from mouse, the most extensively studied rodent is rat. The organisation of the rat MHC (RT1) is like that in the mouse. In the RT1 complex six MHC loci have been identified A, B, D, E, G and C which are arranged in this order. The *RT1.A* and *RT1.E* loci are classical class I loci homologous to mouse H-2D and H-2K and human HLA-B and HLA-A loci respectively. *RT1.A* is the main polymorphic locus with 12 alleles, but only two alleles have been detected for *RT1.E* (Misra et al. 1985). The level of functional polymorphism of classical class I antigens detected serologically in the rat is very low (Gill et al. 1983) whereas in

the mouse approximately 100 alleles have been reported for the K and D loci (Steinmetz et al. 1982b). The rat equivalent of the mouse Q/T region is called *RTI.G/C* (Kunz et al. 1989). The *RTI.C* locus has been defined by skin graft rejection and by serology, and it has three alleles which have been designated by the haplotypes of strains in which they were discovered (*a,l,u*). The *RTI.G* locus has two expressed alleles designated as *a* and *b* and a blank *c* allele. Several class I genes have been mapped to the *RTI.C* region and most seem to be of the class Ib type (Lutz et al. 1994). Rat M genes, like the mouse M, have also been found (Gill, 1994). *RTI.B* and *RTI.D* are class II loci corresponding to mouse H-2A and H-2E loci, respectively, and each encodes α and β chains.

In rat, antigens encoded by different class I genes of the same haplotype are homologous. This has been shown in the A^u and E^u antigens which are the product of the two different loci but which differ only by 1-3% whereas the A^u and Aⁿ antigens encoded by alleles of the same gene differ by about 30% (Misra et al. 1987). This finding is significant in the light of the restricted polymorphism of the rat MHC antigens. The *RTI.A* locus encodes both membrane bound (45kD) and soluble (40kD) class I molecules. The soluble form is homologous to the murine Q10 gene product and is generated as a result of alternative splicing which removes the transmembrane domain (exon 5) and possibly exons 6 and 7 (Singh et al. 1988). Rat also differs from the mouse in terms of having two major allelic forms of the TAP2 genes, differing by 25 amino acids. Since the rat has only one major expressed class I locus, the different TAP alleles may provide an alternative way of diversifying the immune repertoire (Howard, 1991).

The lagomorph MHC (rabbit) shows considerable homology in gene number and

organisation with HLA. The class I region contains between 8 and 13 genes. The cDNA clone pR9 was isolated from the rabbit cell line RL-5 and conforms to the typical class I structure (Tykocinski et al. 1984). A genomic class I clone has also been sequenced (Marche et al. 1985), and this clone displays the characteristic 8 exon class I gene structure. In the class II region, DR, DP, DQ1, DQ2 and DO homologues have been identified (LeGuern et al. 1985; Sittisombut and Knight. 1986). Rabbit *DPA* and *DPB* genes have also been sequenced and appear intact (Sittisombut et al. 1988).

1.12 Class I MHC genes from domesticated animals

Although the MHC of man and mouse are best characterised and understood, cloning of MHC genes from other species will provide additional insights into the evolutionary relationships of these genes and expose any features which are atypical. Domestically important species are particularly relevant in this type of approach because the results of investigations of smaller animals e.g. mice, rats and rabbits, may not always be directly applicable to the immunology of larger animals. This is reflected in studies of MHC influence on disease resistance/susceptibility, because each species has its own particular disease repertoire.

Ungulate MHCs have been examined in cattle, sheep, pigs and horses by the application of Southern blotting hybridisation. All of the above species have multiple class I hybridising sequences. A striking feature of the swine MHC (SLA) class I region is that it contains only seven genes (Singer et al. 1988). Two swine genomic clones PD1 and PD14 show all the features attributable to class I genes and have been expressed in L cells (Singer et al. 1982; Satz et al. 1985). PD1 has been introduced into mice as a transgene (Frels et al. 1985) and shown to function in graft

rejection. Singer's group has isolated and sequenced five of the seven genes and on the basis of sequence homology they fall into three different groups. However, there is evidence for only two class I loci in pigs (Lunney et al. 1988).

The horse MHC (ELA) is located on chromosome 20 and contains up to 30 class I genes (Alexander et al. 1987). Serological analysis has identified 19 specificities at a single class I locus whereas biochemical studies of horse class I indicates that there are at least two expressed genes (Donaldson et al. 1988). It has been also reported that horses have soluble class I antigens in their serum (Lew et al. 1986) at levels comparable to that of mouse Q10 (Mellor et al. 1984). Recently, Ellis and co-workers (1995) have reported that four class I genes are transcribed in the horse based on the number of different sequences identified.

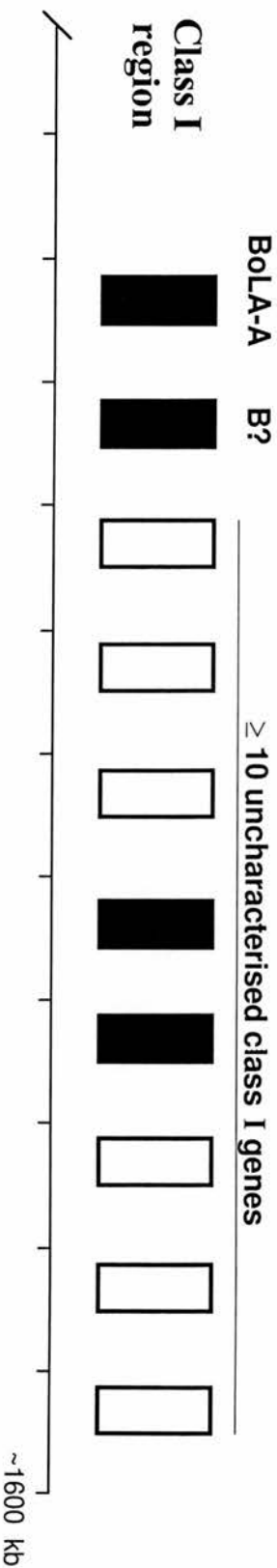
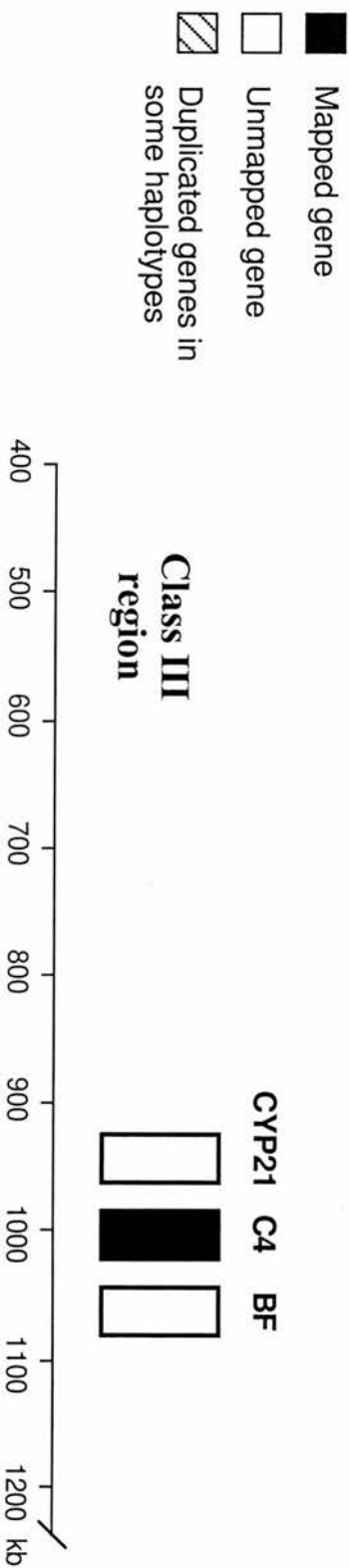
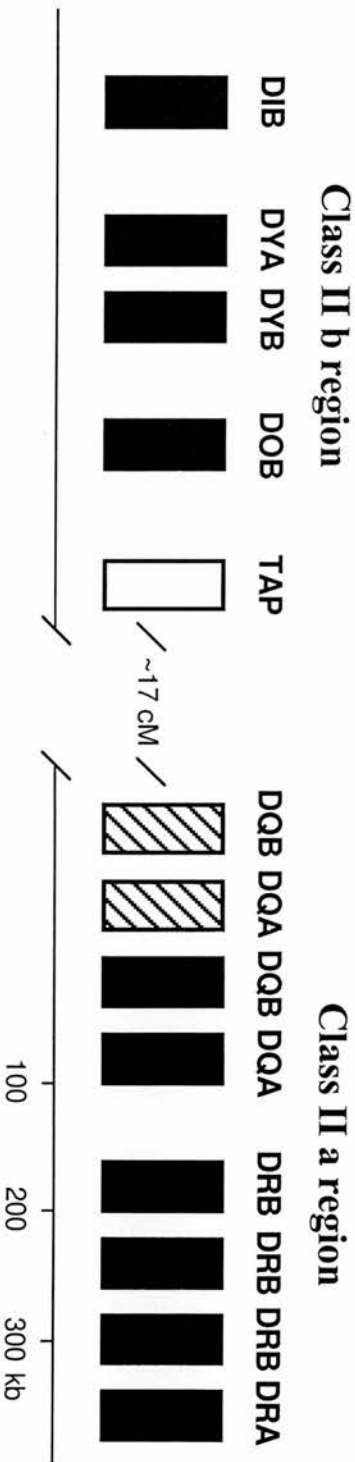
The sheep MHC (OLA) has been shown to contain three expressed class I genes by cDNA cloning experiments and biochemical studies (Grossberger et al. 1990; Puri et al. 1987). However, it is not known if all three genes are polymorphic. Like cattle, sheep MHC class II region also contains duplicated *DRB*, *DQA* and *DQB* loci (Andersson and Davies, 1994).

1.13 The bovine MHC

The bovine MHC (BoLA), is located on the short arm of chromosome 23 (Fries, 1986), and its general organisation is very similar to human. A tentative map of the bovine MHC is shown in Fig 1.3. The map has been drawn partly on the basis of gene order established in man, mouse and rat (Trowsdale et al. 1991) as in the cattle the exact order of the genes has not yet been completely determined. The BoLA complex is divided into four regions: class I; class IIa, which encodes the functional

Fig. 1.3 A tentative map of bovine major histocompatibility complex.

Map of the bovine MHC



class II molecules; class IIb, which harbours a number of class II genes whose function is not yet known; and class III, which contains a large number of genes several of which encode proteins of immunological function. The class I region is the most telomeric part of the MHC and the class III region is located between class I and class IIa. The class IIa region is tightly linked to class I region (Usinger et al. 1981; Lindberg and Andersson, 1988). The class IIb region is separated from the class IIa region by a recombination distance of approximately 17cM (Andersson et al. 1988; Van Eijk et al. 1993). The class I, class III and class IIa loci are generally considered as a genetic unit and are inherited *en bloc*.

1.13.1 *Discovery of bovine class I loci*

The class I antigens of bovine MHC were identified by Spooner et al. (1978) and Amorena and Stone (1979) on the basis of the skin grafting experiments and serology. The class I molecules in cattle have so far only been analysed by serology using alloantisera, which are polyspecific. Class I alloantisera have been obtained from cows (Spooner et al. 1979a; Amorena and Stone, 1982), since a maternal immune response to paternally inherited foetal MHC antigens can occur during pregnancy (Redman et al. 1987). However, methods more reliable for the production of class I alloantisera are by immunization with leucocytes, or by subcutaneous skin implantation (Spooner et al. 1978; Amorena and Stone, 1982). Alloantisera obtained by immunization usually give higher antibody titers than sera obtained from parous cows. The allo-antisera were tested against a panel of cells and absorbed to reduce their cross-reactivity, and produce "operationally monospecific" allo-antisera that are thought to recognise single BoLA specificities. These allo-antisera, whether against class I or class II, are employed in a microcytotoxicity assay to determine the different specificities expressed in a

particular animal.

A series of international comparison tests conducted in five workshops using serology typing, defined a total of 53 class I specificities which behave as alleles of a single polymorphic class I locus (Davies et al. 1994). Twenty-seven specificities have been assigned to the BoLA-A locus, while 26 are still classified as workshop (denoted by "W") specificities. The assignment of the serological specificities to one locus was based on the observation that individual animals express a maximum of two specificities, with no evidence of recombination (Bull et al. 1989; Bernoco et al. 1991). However, a high frequency of null alleles (undefined) in many cattle breeds shows that a considerable number of bovine MHC class I gene products have yet to be identified. As more typing reagents become available, the antigens presently characterised will, in a number of cases, be 'split', resulting in the detection of epitopes which are characteristic of individual gene products. Such a splitting of A locus antigens has been seen with the W6 specificity (Spooner and Morgan, 1981). Marked differences in BoLA gene frequencies are seen between European breeds (Oliver et al. 1981), and also between breeds throughout the world including the tropics (Kemp et al. 1988). Interestingly, local specificities can be found which are defined by sera that do not react well with the samples of other breeds, suggesting breed-specific influences on polymorphism. It should be noted at this point that most of the animals tested in BoLA workshop have a similar genetic Background (*Bos. taurus*) with only 7.7% of the animals in the third workshop representing *Bos indicus* and only 6.6% were *B.taurus* x *B.indicus* crosses (Bull et al. 1989). This, coupled with the fact that most of alloantisera are produced in *B.taurus* animals, draws attention to the possible bias that this may incur in serological studies with non-european animals.

The complexity of many BoLA class I reagents has stimulated considerable debate about whether BoLA class I typing sera detect alleles at a single locus or are haplotype-specific, and reacting with the products of more than one polymorphic class I gene. Stear and co-workers (1982) have shown that some distinct locally-defined antigens which were inherited together in some families, may represent the products of a second locus. This could be for two reasons tight linkage of class I loci may result in sera identifying haplotypes, rather than individual products. The sera produced in cattle may also identify only the most antigenic locus.

Although alloantisera have been the basic tools for bovine class I antigen definition, monoclonal antibodies have the advantage that they see individual epitopes and that they can be produced in large quantities. Several monoclonal antibodies (mAbs) detecting epitopes on class I antigens of other species have been tested with bovine cells, but most detect non-polymorphic determinants (Brodsky et al. 1981). Only a limited number of mAbs which define bovine class I polymorphism have been reported (Spooner and Pinder, 1983; Teale et al. 1986).

The number of the expressed bovine class I loci has also been addressed using biochemical techniques such as 1D-IEF, which has greater resolution than serology. This method, when used with anti-HLA class I monoclonal antibodies that cross-react with bovine class I molecules, produces multi-band patterns (2-4) from each haplotype, some of which can be assigned to serological specificities (Joosten et al. 1988; Oliver et al. 1989; Al-Murrani et al. 1993). Each haplotypic pattern observed was unique, but there were bands that were shared between patterns. Whether these bands represent identical products from a particular locus or distinct products that focus at the same pI cannot be determined at present. These IEF banding patterns

may reflect the expression of several bovine class I loci at the protein level. Using 1D-IEF, these investigators have identified serologically undetected (blank) haplotypes, BoLA-A locus polymorphisms (splits) that were not resolved using the available serological reagents, and the products of more than one expressed class I gene. Joosten and co-workers (1992) detected subtypes of the serologically-defined A10 specificity in African Boran cattle. The subtypes appeared to be associated with different second locus products. Therefore, they concluded that serologically-defined A10 haplotypes encoded at least two independent class I locus products, expressed on normal bovine PBM. Similar subtypes have been reported for HLA-B27 and HLA-A2 (Lopez de Castro, 1989). In the Fifth BoLA workshop, IEF typing was included to improve and support the serological definition of class I antigens by detecting three subtypes of the A15 (A8) serotype and two subtypes of A22 (w49) (Davies et al. 1994).

1.13.2 *Class I region*

The class I region spans several hundred kilobases (kb) (Bensaid et al. 1991), and southern blot analysis using a human class I cDNA probe indicated the presence of multiple class I genes, possibly 20 or more (Lindberg and Andersson, 1988). There is some uncertainty about the number of class I genes present in cattle, and little is known concerning the number of the class I genes expressed. The presence of two or three expressed class I genes is supported by cDNA cloning experiments (Ennis et al. 1988; Bensaid et al. 1991; Ellis et al. 1992). The presence of at least three expressed class I loci was supported by Garber et al. (1993), based upon comparison of their cDNA sequences with those published previously. Recently, Garber et al. (1994) isolated six different class I cDNAs from one heterozygous animal and suggested the expression of at least three class I genes. However, these clones were

not transfected to determine the number and nature of the expressed products from this animal. Expression of more than one class I gene by a single haplotype has also been demonstrated by the use of monoclonal antibodies. The independent nature of two loci was demonstrated by the differential reactivity of monoclonal antibodies with class I molecules on mouse L cells transfected with DNA from a MHC homozygous cow (Toye et al. 1990). The cDNA clones from the same haplotype isolated by Bensaïd and co-workers (1991) were shown to be different locus products by differential hybridization of their 3'-untranslated regions to the L cell transfectants. The 3'-untranslated regions from these clones were also used as locus -specific probes to map the two loci. The two genes were shown to be less than 210kb apart. This evidence suggests that the two loci were tightly linked. To resolve the number of expressed class I genes in cattle, it is important to combine cDNA cloning studies with the investigation of expression of individual class I gene products by transfection, and then to correlate the functional serological specificity with the nucleotide sequence.

1.13.3 *Class II region*

Using human probes, Andersson and Rask (1988) extensively analyzed the BoLA class II region. They concluded that this region includes homologues of the human DR and DQ genes. In the class IIa region there is one *DRA* gene and three *DRB* genes, one of which is highly polymorphic. In contrast to the mouse and human MHCs, in which only the DR-like genes vary in number, in cattle the number of DQ genes vary from two to four in different haplotypes, while the number of DR equivalent genes appears constant. One arrangement consists of single *DQA* and *DQB* genes, a second contains two *DQA* genes and a single *DQB* and the third has two of each type.



1.13.4 *Class IIb region*

Southern blot analysis has revealed the presence of *DOB*, *DNA*, *DYA*, *DYB* and *DIB* genes (Andersson et al. 1988; Andersson and Rask, 1988). These genes do not appear to be highly polymorphic, and there is no evidence of their expression at the cell surface. In addition no evidence of DP-like genes has been found, which suggests that they have either diverged considerably from their human counterparts, or that they have been deleted, as in the mouse (Andersson and Rask, 1988).

In the class III region of cattle, only the CYP21, C4 and Bf genes have been mapped by linkage analysis (Andersson et al. 1988; Teutsch et al. 1989, 1990).

1.14 **MHC and disease association**

The capacity of a given MHC molecule to bind a peptide and to form a complex is determined by the sequence of its antigen binding site. In other words, the MHC alleles carried by an individual will influence whether that individual can mount an appropriate immune response to a given antigen, be it self or non-self derived. Thus, a particular MHC allele or haplotype might confer susceptibility or resistance to a certain infectious disease, but may be protective against other agents. MHC polymorphism is an adequate answer for a population to the problems caused by many diseases: some individuals will be sacrificed because they succumb to certain agents, but the majority may survive and develop immunity. The relevance of MHC polymorphism to the regulation of the immune response has stimulated many researchers to study the association between disease and MHC. In man, numerous diseases (autoimmune, immune complex mediated and non-immune) are shown to associate with particular HLA alleles or haplotypes (Todd et al. 1987). Some of these HLA-associated diseases are: ankylosing spondylitis, coeliac disease, juvenile

diabetes mellitus, Graves disease, systemic lupus erythematosus, rheumatoid arthritis. In addition, susceptibility to typhoid, yellow fever and malaria is HLA associated (Piazza et al. 1972, De Vries et al. 1976, 1979, 1989; Hill et al. 1991). One reason for undertaking studies of the BoLA system is that it may provide a means for selecting more productive or disease resistant cattle, by identifying BoLA types which are associated with good characteristics, or selecting against those associated with bad traits. Various associations between BoLA (mainly class I serotypes) and a number of diseases have been reported. However, most of these studies relied on statistical analysis and not on any immunological or biochemical data. The first report of MHC-disease associations in cattle came from Solbu et al. (1982), who analyzed the effect of BoLA class I polymorphism on mastitis among cows of the Norwegian Red breed. They found that the BoLA type W2 was associated with resistance, while w16 was associated with susceptibility. These data were later confirmed (Larsen et al. 1985; Solbu and Lie, 1990; Vage et al. 1992). Other MHC disease association studies in cattle include studies on bovine leukaemia virus (BLV) infection, bovine viral diarrhoea, endo and ectoparasite. Lewin and Bernoco (1986) were first to provide evidence that the subclinical progression of BLV is influenced by genes in the BoLA complex. They demonstrated that in a herd of short horn cattle, resistance to B-cell proliferation in BLV infected cows segregated with w4 and w7-bearing haplotypes and susceptibility with w26 bearing haplotypes.

In addition to MHC disease associations, MHC effects on other traits like ovulation rate, body weight and milk production have been proposed (Hines et al. 1986; Batra et al. 1989). However, these studies indicated weak associations which were difficult to confirm. Evidence of the involvement of MHC compatibility in

reproduction has also been presented (Joosten et al. 1991). They hypothesised that MHC incompatibility between dam and calf would facilitate the expulsion of the placenta. In accordance with this class I compatibility might be associated with retention of placenta (Joosten and Hensen, 1992).

1.15 Aims

Most of the knowledge about the MHC of cattle (BoLA), over the first decade of its study has come from serological studies. However, in the last few years much has been done to employ more sensitive methods in the study of the BoLA system, although they represent only the first steps along a long path towards a clear understanding of the bovine MHC.

Serological studies, which represent the vast majority of investigative studies on the BoLA system, have indicated the presence of a single highly polymorphic locus encoding class I molecules. However, other biochemical and molecular biological studies have indicated that the system is much more complex than is suggested by serology alone. The main thrust of this project was to apply molecular biological approaches to the analysis of class I genes in cattle. The aim of the project was to dissect individual functional class I expressed products from a heterozygous animal, class I typed as BoLA- A10/A11, by transfection and characterisation of a series of bovine class I genomic clones. The transfected cell lines expressing individual class I molecules were used for the characterisation of the expressed products. Lastly, in order to determine how many different functional class I genes were expressed from this haplotype, the nucleotide sequence coding these expressed molecules was determined and compared with each other and the other bovine published cDNA sequences.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

The chemicals used during the research for the project were supplied by Sigma Chemical Co., Poole Dorset, UK, or Fisons Scientific Equipment, Loughborough, UK unless stated otherwise. Radiolabelled compounds were supplied by Amersham International PLC, Amersham, UK. Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (NEB), UK or Boehringer Mannheim, BCL, Lewes, UK. Tissue culture medium was purchased from Life Technologies, Renfrewshire, UK. Recipes for solutions used during this project are given in Appendix 2A and B.

2.2 Bacterial cultures

2.2.1 *Bacterial growth media*

The basic bacterial growth medium was Luria Bertani (LB) broth. This medium was supplemented with the antibiotic ampicillin at 100 μ g/ml for selection of transformed cells, since all of the plasmid vectors used during this project contained the gene β -lactamase which confers ampicillin resistance. LB agar /plates were made with LB + 1.5% (w/V) bacteriological agar. These plates were used with bacteria transformed with pBR322 derivatives. In this system, transformed cells were selected on plates containing ampicillin. Solid or liquid NZY medium was used for propagation of phage.

Liquid NZY broth was supplemented with 0.2% Maltose, and MgSO₄ was added to 20mM just before pouring the plates.

2.2.2 *Bacterial strains*. *E.coli* strains used during this project. are given in Table 2.1

Strain	Genotype	Uses	Reference
DH5 α	F', Φ 80 <i>lacZ</i> Δ 15	Plating and growth of Plasmids.	Hanahan (1983)
Y1088	<i>e14</i> ⁻ (<i>mcrA</i>), Δ (<i>lac</i>)/U169, <i>supE</i> , <i>supF</i> , <i>hsdR</i> , <i>metB</i> , <i>tpR</i> , <i>tonA21</i>	Propagation of EMBL3	Young and Davis (1983)

The Φ 80d *lacZ* Δ M15 marker provides complementation of the β -galactosidase gene fragmentation on PUC or similar vectors and, therefore, can be used to for blue/white screening colonies on bacterial plates containing X-Gal.

2.2.3 Preparation of plating bacteria

The host strain used for propagation of phage λ EMBL3 clones was Y1088. 50ml of broth supplemented with 0.2% maltose was inoculated with a single bacterial colony. The culture was grown with shaking at 37°C until the culture optical density at 600nm reached 0.5. The cells were then harvested by centrifugation at 3000rpm for 10 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in 20ml of sterile 0.01M MgSO₄. The preparation of plating cells was stored at 4°C and could be used up to 3 weeks.

2.3.Preparation of phage DNA

A single colony of *Escherichia coli* Y1088 was inoculated into 100 ml NZY broth in a 500ml conical flask. The culture was incubated at 37°C for 5-6 hours with vigorous shaking. The optical density (OD) was read at 600nm. Cell concentration was calculated assuming that 1 OD 600 = 8 x 10⁸ cells/ml (Sambrook et al. 1989). Aliquots of 10¹⁰ cells were centrifuged at 3000rpm for 10 minutes. The supernatant

was discarded and cells were resuspended in 3ml of phage buffer (SM Buffer). An aliquot of 5×10^7 pfu of phage stock was adsorbed to 10^{10} plating cells at 37°C for 20 minutes before being added to 500ml of NZY broth, prewarmed at 37°C in a 2L flask. The culture was vigorously shaken until lysis occurred i.e approximately after 6 hours. To induce lysis, 10ml of chloroform per 500ml of culture was added and the culture was incubated at 37°C for 10 minutes. The lysed culture was cooled to room temperature and DNAase and RNAase were added to final concentrations of 1µg/ml. The lysate was left at room temperature for 30 minutes before the bacterial debris was removed by centrifugation at 10,000rpm for 10 minutes at room temperature. Polyethylene glycol 6000 (PEG 6000) 10% (w/v) and NaCl (1M) was added to the supernatant which was left 4°C for at least 1 hour. The precipitated phage was pelleted by centrifugation at 10,000 rpm for 30 minutes at 4°C. The phage pellet was dissolved in 1.5ml of SM buffer and layered on a CsCl step gradient comprising 1ml of 1.7g/ml CsCl, 1ml of 1.5g/ml CsCl and 0.75 ml of 1.3g/ml CsCl. The gradient was centrifuged in a Beckman Sw50 rotor for 2 hours at 38000 rpm and 4°C. The bluish phage band was collected by puncturing the side of the tube with a 21 gauge needle and 1ml syringe. The phage were dialysed for two hours against two changes of 10mM NaCl, 50mM Tris HCl pH 8.0, 30mM MgCl₂ at room temperature. The dialysed phage particles were used for the extraction of DNA. DNA from the phage particles was extracted with an equal volume of 1:1 v/v phenol: chloroform followed by an equal volume of chloroform alone. Each extraction was thoroughly mixed for 10 minutes and spun at 3000rpm for 10 minutes to separate the phases. The aqueous phase from the last extraction was dialysed overnight against 2 x 2l of TE pH 8.0. It was then analyzed on 0.7% agarose gel to check the size and quality of preparation. The concentration of the DNA was estimated by spectrophotometry as described in section 2.6.5.

2.4 Preparation of plasmid DNA

2.4.1 *Small-scale plasmid DNA minipreps*

Small-scale purification of plasmid DNA was performed using Wizard Minipreps DNA purification system (Promega). *E.coli* cultures containing plasmid (1-3ml) were grown in LB medium + ampicillin (100 μ g/ml) at 37°C overnight with shaking and 1.5 ml aliquots were harvested in microfuge tubes. The cell pellets were resuspended in 200 μ l of cell resuspension buffer, lysed by cell lysis solution, and lysates were mixed by inversion and left on ice for 5 minutes. Once the cells were disrupted, high molecular weight DNA and proteins were precipitated by addition of 200 μ l of neutralization solution (pH 5.0). The debris was pelleted by centrifugation for 5 minutes. Approximately 600 μ l of the supernatant was removed to fresh tubes. To this, 1 ml aliquots of resin mix were added and mixed by inversion. The mixture was poured into 2ml syringe barrels attached to minicolumns, and allowed to run through by gravity. The columns were washed with 2ml 80% isopropanol, and dried by spinning for 20 seconds at 12000rpm. The plasmid DNA was then eluted in 50 μ l of TE by spinning for 20 seconds at 12000rpm.

2.4.2 *Large-scale preparation of plasmid DNA for double stranded sequencing*

The method used for preparation of plasmid DNA from 200ml cultures was essentially the same as that described for plasmid minipreps, up to the preparation of cell lysates except that volumes were scaled up for larger quantities. The purification of plasmid DNA was performed using Qiagen tip 500 as per manufacturer's instructions. The cleared lysate was loaded on pre-equilibrated Qiagen-tips by gravity flow. The Qiagen tips were then washed with 1M NaCl wash buffer (QC) for removal of contaminants. DNA was eluted from the Qiagen-tips with 15ml of elution buffer (QF) 1.25M NaCl at (pH 8.5). The eluted plasmid DNA

was precipitated with 0.7 volumes of isopropanol by centrifuging at 10000 rpm at 4°C. DNA pellets were washed with 70% ethanol to remove traces of salt. After briefly air drying, the purified DNA was resuspended in 100µl of TE, and was ready for use in transfection, sequencing and cloning.

2.5 Preparation of genomic DNA

Genomic DNA was prepared by the salt extraction method of Miller et al. (1988). Fresh or frozen PBL (10^7 cells/ml), or cells from 10ml of blood, were mixed with 40-45ml lysis buffer (0.3M Sucrose, 1M Tris-HCl pH 7.5, 1M $MgCl_2$, Triton-X-100) in a 50ml tube, and left on ice for 10 minutes to allow the lysis of RBC's. Remaining lymphocytes were pelleted at 2500rpm for 10 minutes at 4°C, and resuspended in 9ml of solution B (0.5M NaCl/0.5M EDTA) after discarding the supernatant. To this 0.5 ml of proteinase-K/SDS solution from a stock of 8mg/ml proteinase-K, 10% SDS was then added. The mixture was incubated at 50°C overnight. 2ml of saturated NaCl was then added to the lysate, and shaken vigorously for 15 seconds. After centrifugation for 10 minutes at 3000rpm, the supernatant was poured into a new tube, and volume was measured. If the supernatant was not clear, this step was repeated. DNA was precipitated by adding two volumes of ice cold 100% ethanol and mixing by inversion. The DNA precipitate was removed with a pipette, washed in 70% ethanol, vacuum dried and resuspended in TE.

2.6 Purification and quantitation of DNA

2.6.1 Gel electrophoresis of DNA

DNA fragments were analyzed on 0.8-1.5% w/v agarose gels containing 0.5µg/ml ethidium bromide. TAE buffer was used when it was necessary to purify DNA from

the gel, since TBE buffer can reduce efficiency of DNA recovery, and may inhibit subsequent enzyme manipulation. TBE buffer was used on all other occasions. Mini-gels generally used 1-1.5% agarose and were run at 10-12 volts/cm. Large 20cm gels were cast at 0.8% concentration and run either slowly overnight at 1-2 volts/cm or during the day at 3-6 volts/cm. Preparative gels used either ultrapure agarose or low melting temperature agarose (Nusieve GTG, FMC, Bioproducts) at appropriate concentration and were run overnight at 1-1.5volts/cm. All samples contained 0.2 volumes of DNA loading dye (3% Ficoll, 0.1%SDS, 100mM Na₂EDTA pH 8.0, and 1.2mg/ml of Bromophenol Blue). *Eco* RI, *Hin* d III digested lambda DNA markers were included on gels for size estimation.

2.6.2 *Non-denaturing Acrylamide gels*

All acrylamide gels (Acrylamide /N,N',-methylenebisacrylamide, 29:1) were cast as 1.0mm thick gel using BioRad gel casting apparatus. The percentage of acrylamide was selected depending upon the sizes of fragments to be separated. These gels were run at approximately 30 watts. Once the electrophoresis run was complete the gels were stained with ethidium bromide at the concentration of (0.5µg/ml in 1xTBE) for 30 minutes, and viewed by UV transillumination.

2.6.3 *Purification of DNA fragments*

DNA fragments were purified from agarose gel using Spin-X tubes (Costar). Bands, visualised by UV illumination were excised in small agarose blocks and transferred to separate Spin-X tubes. The tubes were placed at -20°C for 30 minutes to freeze the agarose blocks, and DNA was eluted from the agarose by spinning the tubes at 12000rpm for 20 minutes. In some cases the Wizard DNA clean-up system (Promega) was used to purify DNA from restriction or modification reactions.

2.6.4 *Ethanol precipitation of nucleic acids*

This method is used to concentrate DNA and RNA. Ethanol precipitation of nucleic acids involved adding 2.5-3 volumes of 100% ethanol with 0.3 volumes of 3M sodium acetate pH 5, mixing and incubating at either -20°C or -70°C for periods of 3 hours or 30 minutes respectively. The DNA or RNA could then be recovered by centrifugation followed by removal of the ethanol supernatant. Excess ethanol was then evaporated either at room temperature or in a vacuum drier. The pellet was then dissolved in the appropriate buffer and stored at either 4°C (genomic DNA samples only), -20°C (DNA samples) or -70°C (RNA samples only).

2.6.5 *Spectrophotometric analysis of DNA and RNA samples*

The concentration of DNA in a purified sample was generally estimated by running a small aliquot on a 0.8-1.5% w/v agarose gel, and examining the intensity of the fluorescence under UV illumination. The concentration of DNA in larger preparations was estimated from its absorbance at a wavelength of 260nm (OD_{260}). An OD_{260} of 1.0 is roughly equivalent to 50µg/ml double stranded DNA, or 40µg/ml single stranded DNA or RNA. The $OD_{260/280}$ ratio gave an estimate of the purity of the sample, where a ratio of 1.8:1 indicated a sample free from significant protein or phenol contamination (Sambrook et al. 1989).

2.7 **Preparation of plasmid and insert DNA for cloning**

2.7.1 *Preparation of plasmid vector for cloning*

3-5µg of plasmid vector was digested with 5-20 units of the required restriction enzyme(s) in a total volume of 20µl, using the manufacturer's buffer, normally for 1-3h at the recommended temperature. If the vector ends could self-ligate then they were dephosphorylated by the addition of 1-2 units of calf intestinal phosphatase

(CIP; Boehringer Mannheim), followed by incubation at 37°C for 30 minutes. Inactivation of CIP was achieved by using the Wizard DNA clean-up system (Promega). DNA was eluted in 20-30 μ l of TE and analyzed by gel electrophoresis.

2.7.2 Preparation of DNA fragments for blunt ended cloning

DNA fragments from PCR were prepared for blunt-ended cloning using T4 DNA polymerase (Boehringer Manneheim) to "fill in" recessed 3' ends, and T4 polynucleotide kinase (New England Biolabs) to phosphorylate the 5' ends. The DNA fragments were incubated at 37°C for 30 minutes with ATP and 10 units of kinase in the manufacturer's buffer in a total volume of 30 μ l. For the filling-in reaction, approximately 10 units of T4 DNA polymerase were added along with 100 μ M of each dNTP, and 2 μ l of 10x buffer to give a total volume 50 μ l, and the incubation was continued for a further 30 minutes. DNA was purified using the Wizard Clean up system, and the DNA fragments eluted in 20 μ l of TE.

2.7.3 Ligation of DNA fragments

Ligations were carried out using 100ng of vector DNA with the insert DNA present at threefold molar excess. Ligations were generally performed in 15 μ l volumes containing 2-4 units of T4 DNA ligase (New England Biolab) and the manufacturer's buffer. Ligation reactions were incubated at 16°C overnight. In the case of TA cloning of PCR products the manufacturer's instructions for ligations were followed (Invitrogen). In addition, appropriate controls ligations were set up to assess ligase, CIP and restriction enzyme.

2.7.4 Transformation of ligations

Subcloning efficiency DH5 α competent cells (Life Technologies) were thawed on ice, and used as per manufacturer's instructions. Cells from 50 μ l transformations were plated out on LB + Ampicillin agar plates as described in section 2.2.1. Plates were incubated at 37°C for 16 hours, and single colonies were picked for analysis of plasmid DNA.

2.8 DNA Sequencing

2.8.1 Sequencing of double standard templates

Prior to double stranded sequencing, the DNA template was denatured and annealed to the sequencing primer. 3-5 μ g of double stranded DNA in 18 μ l of H₂O was denatured by adding 2 μ l of 2M NaOH/2mM EDTA and incubating at room temperature for 5 minutes. The denatured plasmid DNA was neutralised by adding 2 μ l of 3M NaAcetate pH 4.5. To precipitate the DNA 2-3 volumes of 100% ethanol were added, mixed and tube was kept at -20°C for 20 minutes. DNA was pelleted by centrifuging at 12000rpm for 20 minutes. The pellet was washed with 70% ice cold ethanol, dried and resuspended in 7 μ l of distilled H₂O and was immediately used in annealing reactions by adding 2 μ l of 5x sequenase buffer and 1 μ l of primer (1 pmol/ μ l). The annealing mixture was incubated for 2 minutes at 65°C, and then cooled slowly to room temperature.

2.8.2 Sequencing reactions

DNA sequencing reactions were generally performed by the dideoxy chain termination method of Sanger and co-workers (1977). Most reactions were carried out in eppendorf tubes using the Sequenase Version 2.0 kit (USB Corporation) with the supplied Sequenase Version 2.0 T7 DNA polymerase and α -³⁵S dATP

(Amersham: 3000Ci/mmol) according to the manufacturer's protocol.

2.8.3 *Cycle sequencing*

The sequencing of double stranded PCR products was performed using Thermal Cycle-sequencing kit (New England Biolabs) as per manufacturer's instructions. To improve the quality of sequence data, the PCR products were first purified by the Wizard DNA clean-up system. This system was also used, to obtain DNA sequencing information from regions which had a high degree of secondary structure and were not resolved by the dideoxy chain termination method.

2.8.4 *Sequencing gel electrophoresis*

DNA sequencing gels were composed of 4.5% or 6% (W/V) acrylamide (29:1 acrylamide:N-N methylene bisacrylamide), 8M urea (Sigma; molecular biology grade), in 1x TBE buffer. The gel casting plates 21cm x 50cm x 0.4mm and sequencing from BioRad Sequi-gen were used throughout with 1xTBE as the running buffer in upper and lower reservoirs. Before loading samples, excess urea was washed out of the wells, using a needle and syringe. The sequencing samples were denatured by heating at 85°C for 3-5 minutes, and 3 μ l of each sample was loaded onto the gel. Gels were run for 3-5 hours at 50 watts. Sequencing gels were dried on to Whatman no. 3 paper at 80°C under vacuum on a BioRad gel drier. Gels were exposed to AGFA Curix X-ray film for 2-4 days at room temperature.

2.8.5 *Analysis of sequence data*

Sequence data was analyzed using version 7 of the University of Wisconsin Genetics Computer Group Sequence Analysis software (Genetics computer group, 1991).

2.9 RNA preparation

2.9.1 RNA isolation

For RNA work all glassware, tubes and aqueous solutions were autoclaved. Total RNA was isolated with RNazol solvent (Biogenesis labs, Poole, England), using a modified version of manufacturer's instructions. Cells were spun for 5 minutes at 10000rpm and the pellet was thoroughly solubilised in 1ml of RNazol. 100 μ l chloroform supplemented with 4% iso-amyl alcohol (Fisons) was added and the samples vortexed for 15 seconds. The tubes were incubated for 10 minutes on ice before spinning for 15 minutes at 14000rpm in a refrigerated microfuge at 4°C.

The upper aqueous layer (approximately 500 μ l) was then removed from the tube to a fresh sterile eppendorf tube, taking care not to transfer any of protein interface. RNA was then precipitated by adding 800 μ l ice cold 100% ethanol. The pellet was centrifuged as before for 10 minutes, and the ethanol wash was repeated. After the second ethanol wash, the pellet was dried under vacuum for approximately 5 minutes before resuspension in a small volume (10-50 μ l) of autoclaved distilled H₂O. The amount of RNA obtained was determined by spectrophotometry as described earlier.

2.9.2 RNA formaldehyde gel electrophoresis

The integrity of isolated total RNA was determined by agarose gel electrophoresis under denaturing conditions (Sambrook et al. 1989). Gels were run using MOPS buffer (appendix 2A). 5-10 μ g of RNA in 12 μ l of dH₂O was prepared for electrophoresis by adding 5 μ l 10x MOPS, 8 μ l paraformaldehyde, 25 μ l deionised formamide and 1 μ l of 10mg/ml ethidium bromide, and heating to 55°C for 15 minutes. Before loading 3 μ l of loading buffer (section 2.6.1) was added to the samples. Formaldehyde gels were run overnight at 25 mA, and when viewed under

UV illumination, RNA was visualised primarily as ribosomal RNA (rRNA), 18s, 12s and 5s bands. The sharpness of the RNA bands generally indicated that total RNA prepared was of reasonable quality.

2.9.3 *cDNA synthesis*

First-strand cDNA synthesis was carried out by reverse transcriptase using the "Superscript" system (Life Technologies), following the manufacturer's instructions. Optimally, 5 μ g of RNA in 13 μ l dH₂O was used. The reaction was first primed by adding 1 μ l of 500 μ g/ml oligo dT primer, heating to 70°C for 10 minutes and chilling quickly on the ice. The resulting mixture, with the oligo dT bound to the polyA tail of the mRNA, was then reverse transcribed to make cDNA by adding: 2 μ l 10x synthesis buffer, 2 μ l 0.1M dithiothreitol (DTT), 1 μ l 100mM dNTP mixture and 1 μ l 20IU/ μ l reverse transcriptase. After incubating at room temperature for 10 minutes, the mixture was incubated at 42°C for 50 minutes. The reaction was terminated by heating to 90°C for 5 minutes and then chilling on ice for 10 minutes. After a brief centrifugation to collect any condensation, the cDNA was either used immediately in a PCR amplification, or stored at -20°C.

2.10 Tissue culture materials and methods

2.10.1 *Cells and culture media*

A mouse Ltk⁻ fibroblast cell line (Spandidos and Wilkie 1983) was used for DNA-mediated gene transfer of bovine MHC class I genes. The Ltk⁻ cells were maintained in complete tissue culture medium (Tc) comprising RPMI 1640 containing 25mM Hepes (Life Technologies), 2mM L-glutamine (Life Technologies) supplemented with 10% foetal calf serum (FCS) (Meridian address). The cells were passaged using a 1:20 split ratio just prior to confluence (every 3 days). For transfection, cells were

plated at about 5×10^5 cells per 25cm² tissue culture flasks (Life Tech) 24 hours prior to use.

2.10.2 *Preparation of frozen L cells*

Cells were removed from 75cm² culture flasks just prior to confluence and pelleted by centrifugation at 1000rpm for 5 minutes at 4°C. The cells were counted using a haemocytometer. The pellet was resuspended in ice cold freezing solution A (50% FCS, 50% RPMI 1640). An equal volume of ice cold freezing solution B (20% DMSO in FCS) was added dropwise with gentle swirling to give a final concentration of 1×10^7 cells/ml. Aliquots of 1ml in freezing vials were frozen at -70°C and then stored under liquid nitrogen.

2.10.3 *Isolation of peripheral blood mononuclear cells (PBM)*

PBM were separated from venous blood by centrifugation over ficoll-hypaque S.G. 1.077 (Lymphoprep) at 1500g for 25 minutes at room temperature. The mononuclear cells were removed from the interface of the plasma and the ficoll-hypaque, and washed in phosphate buffered saline pH 7.2 (PBS) by centrifugation at 300g for 10 minutes. The cells were washed twice in PBS and once in RPMI-1640 by spinning at 100g for 10 minutes. Finally, the cells were resuspended in tissue culture medium, counted and adjusted to the required cell concentration.

2.11 **Generation of alloreactive cytotoxic T lymphocytes**

Alloreactive cytotoxic T cell lines were generated which were specific for the BoLA A10 and A11 class I antigens of donor animal from which the genomic library was prepared. An anti-A11 haplotype cell line was generated using a responder animal typed as BoLA class I A3/A10 and an anti-A10 haplotype cell line was generated

from a responder animal typed as BoLA class I A11/A15 in an allogenic mixed lymphocyte culture as described by (Teale et al. 1985). The responder cells were restimulated every week with irradiated peripheral blood mononuclear cells (PBM) from the donor animal. The CD4⁺ T helper cells were removed by complement-mediated lysis with mAb IAH-CC8 (Howard et. al. 1991). The CD8⁺-enriched populations were maintained as continuously-growing cell lines by weekly addition of 10 units/ml of human recombinant IL-2 (Boehringer Mannheim), and stimulation with irradiated PBM from the animal used to prepare the genomic library. The cell lines were assessed with the mAb IL-A51 (Ellis et al. 1986), which is specific for CD8⁺ to ensure that the cell lines were CD8⁺ enriched.

2.12 T cell cytotoxicity assay

The target cells were labelled with 100 μ Ci of ⁵¹Cr sodium chromate in RPMI-1640 at 37°C for 60 minutes with periodic mixing. A standard 4 hour chromium release assay was performed involving the effector (CTL) and target cells in different effector:target ratios (Teale et al. 1985; Spooner et al. 1987). Modifications were made when using the adherent Ltk⁻ and cell lines transfected with phage 19.1 and 4.2. These cells were harvested with EDTA 16 hours before performing the cytotoxicity assay. The cells were resuspended in medium and left in untreated polystyrene tissue culture petri dishes (Sterlin or Corning) to which they do not adhere. The cells were washed twice with medium immediately prior to use in the assay (Townsend et al. 1984) This modification was made because cell death caused by EDTA can interfere with the results. The chromium release assay was also performed for 6 hours instead of 4 hours because fibroblasts are much less sensitive to cytotoxic killing than PBM (Simpson and Chandler 1986). The percentage specific ⁵¹Cr release was used as a measure of target cell lysis and was calculated using the

formula.

$$\% \text{ specific release} = \frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

2.13 Transfection

Plasmid and phage DNA preparations made by Qiagen columns and CsCl gradient methods respectively, (sections 2.3 and 2.6.2) were sufficiently pure to be used for transfection. The cationic lipid DOTAP (Boehringer Mannheim) was used for transfecting mouse Ltk⁻ cells as per manufacturer's instructions. The DOTAP forms unilamellar vesicles (liposomes) in aqueous solution, which spontaneously interact with DNA to form stable complexes. These complexes adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm. Since a very small fraction of recipient cells incorporate DNA in a manner that permits its expression, selection techniques are required to isolate these cells. In the experiments described here, the thymidine kinase (tk) gene of Herpes Simplex Virus was co-transfected with the DNA into cells lacking an endogenous tk gene. Stable transfectants were then selected by survival in HAT medium which is toxic to untransfected Ltk⁻ cells. Expression of a selectable marker after co-transfection often indicates that a cell has also been transfected with the accompanying DNA. The transfections were carried using the following protocol: The Ltk⁻ cells were seeded at 5x10⁵ per flask one day before the transfection. 30μl of Transfectin reagent (DOTAP 1mg/ml) was diluted to 100μl with HBS (20mM Hepes, 150mM NaCl, pH 7.4) in a polystyrene reaction vial. 5μg of DNA was separately diluted to 100μl in

a separate vial. The solutions were mixed and incubated for 10 minutes at room temperature and the mixture was added to the Ltk⁻ cells in Tc medium for 3-24 hours at 37°C, 5-10% CO₂. After transfection the tissue culture medium was replaced and then incubated for a further 48-72 hours. This medium was then replaced with selective medium containing HAT. The HAT media was replaced every 2 days initially and every 3-4 days subsequently as the colonies developed. Transfected cells were harvested with 0.02% EDTA (Sigma) and grown to confluence in 75cm² flasks for further analysis by FACS for class I expression.

2.14 Flow cytometry

Samples of 10⁷ cells were pelleted by centrifugation at 1000rpm for 5 minutes at 4°C, and were resuspended in 100μl of mAb specific for the cell surface marker (1:1000) dilution for 30 minutes. Unbound antibody was removed by three washes in ice cold PBS. After the final wash the cell pellet was incubated with 25μl of FITC-conjugated anti-mouse Ig (1:250 dilution) for 30 minutes. Unbound conjugate was washed off three times and the cells were resuspended in 200μl of PBS for analysis by the FACScan cell analyser (Becton and Dickinson). A list of the various monoclonals used for FACS analysis is given in appendix 2B.

The Lysis II programme was used for acquisition, storage and analysis of the single colour flow cytometric parameters of the labelled cells. The cytometer configurations were calibrated using negative control Ltk⁻ cells stained with appropriate mAb and the conjugates. FITC fluorescence (520nm) emission was detected with photomultiplier tube (PMT) voltage set at 600-620mV. FITC amplification (FL1) was logarithmic (log all 1024 channels). Forward scatter (FSC) and side 90° scatter (SSC) amplifications were linear and set at EOO and 400

respectively. Using FSC verses SSC dot plot, cells were live-gated for acquisition and analysis while cell debris and dead cells were excluded.

2.15 Cell sorting by magnetic activated cell sorter (MACS)

MACS is used to separate cells in suspension according to specific cell surface markers. The cells are labelled on their surfaces with antibodies to which supermagnetic microbeads are coupled. After magnetic labelling, the cells are passed through a separation column which is placed in a magnetic separator. Labelled cells attach to the magnetized matrix in the separation column and are separated from the unlabelled cells which flow through. The magnetic fraction can be eluted and recovered after the removal of the column from the magnetic field. High gradient magnetic cell separation with MACS (Miltenyi et al. 1990) was used for sorting the cells. A total of 1×10^7 cells were used for each sort. Cells were incubated with mouse anti-bovine class I mAb IL-A88 (1:100 dilution) in cold PBS with 2% FCS (MACS PBS buffer) for 30 minutes. The unbound antibody was washed off with PBS. After washing, cells were labelled with IgG2a + IgG2b magnetic beads for 20 minutes at 4°C. Excess beads were washed off with MACS PBS buffer. Cells were then passed through the separation column and were separated from the unlabelled cells. The bound cells on the magnetic matrix in the column were eluted after removing the column from the magnetic field. The positive cells were counted and resuspended in HAT medium for further growth and FACS analysis.

2.16 Micro-lymphocytotoxicity testing

2.16.1 *Target cells*

The adherent Ltk⁻, and transfected cells were harvested from flasks and counted using a haemocytometer. They were resuspended in Hank's Balanced Salt Solution

(HBSS) at a concentration of 2.5×10^5 cells/ml. Ltk- cells were the negative controls and PBM from animal 10769 (class I BoLA type BoLA-A10/A11) provided the positive controls.

2.16.2 *Allo-antisera*

The alloantisera used in this test were produced at the division of Molecular Biology, Roslin Institute from cattle, by reciprocal skin grafting between dam and offspring (Spooner et al., 1979a). These operationally allo-monospecific sera have been used in the five international comparison tests carried out to date (Spooner et al. 1979b; Annon. 1982; Bull et al. 1989; Bernoco et al. 1991; Davies et al. 1994) and have been assigned workshop (A) specificities. The BoLA typing sera were stored undiluted in small aliquots at -20°C or -70°C . Control negative serum, which was selected for non-reactivity with any of the test cells, was included in the lymphocytotoxicity test to estimate the baseline test cell viability.

2.16.3 *Complement*

The source of complement was pooled rabbit sera. Batches of complement were screened for lack of inherent cytotoxicity and for potency in the micro-lymphocytotoxicity test using test cells and antisera of known reactivity. Suitable batches of complement were then stored at -70°C until required in the test.

2.17 **Isoelectric focusing**

The BoLA class I molecules were immunoprecipitated from ^{35}S -methionine metabolically- radiolabelled PBM having the A10/A11 specificity, and also from the 19.1 and 4.2 transfected cell lines. Cells (2×10^7) were centrifuged at 250xg for 10 minutes, resuspended in 2.5ml of methionine-free medium and incubated at 37°C in

an atmosphere of 5% CO₂ for 30 minutes. Radiolabelled ³⁵S-methionine (15mCi/ml, Amersham) was added to a concentration of 20μCi/ml and cells were incubated overnight under the same conditions as above.

2.17.1 Immunoprecipitation of BoLA class I molecules

Labelled cells were centrifuged for 5 minutes at 200xg and washed once by resuspending in 1ml PBS. The sample was then transferred to an eppendorf tube and centrifuged at 16000g for 20 sec. The pellet was resuspended in 1ml of TX114 lysis buffer (0.5% TX114, 50mM Tris-HCl pH 7.4, 5mM MgCl₂ and 1mM PMSF) and incubated on ice for 30 minutes. The cell lysate was centrifuged at 8000xg for 10 minutes at 4°C. The supernatant was transferred to a new tube and incubated at 37°C for 4 minutes. The detergent/aqueous phase were subsequently separated by centrifugation at 300xg for 4 minutes at 37°C and the aqueous phase was discarded. The lower detergent phase (50μl) which contained the bulk of class I molecules was precleared by incubating with 4μl of normal rabbit or mouse serum and 1ml of NET buffer (50mM Tris-HCl pH 7.4, 0.5% NP40, 150mM NaCl, 5mM EDTA and 1mM PMSF). The mixture was incubated on ice for 30 minutes, followed by addition of 75μl "Pansorbin" (Calbiochem, 10% cell suspension of *Staphylococcus aureus*) and again incubated for 15 min on ice. The samples were then centrifuged and the supernatant was transferred to a fresh tube. The last preclearing step was repeated.

For each immunoprecipitation 330μl of labelled precleared lysate and was incubated with 5μl of MHC class I-specific mAb (ascitic fluid) on ice for 75 minutes. The MHC class I/antibody complexes were subsequently precipitated by incubating with 100μl of Pansorbin cells on ice for 30 minutes. Preparations were then centrifuged at 8000xg for 4 minutes, and the supernatant was discarded. The pellet was washed

four times in 0.5ml NET buffer. After the final wash, the pellet was resuspended in 100 μ l of 1U/ml neuraminidase in 50mM sodium acetate, pH 5.5 containing 154mM NaCl and 9mM CaCl₂ (Behring) and incubated at 37°C overnight. A further 0.1U of neuraminidase was added and the incubation was continued for an additional 6hrs. The supernatant was removed and 35 μ l of sample buffer added. The IEF sample buffer contained 9.5M urea, 2% ampholine pH range 3.5-10, 2% NP40 and 5% 2-mercaptoethanol. Samples were incubated at 37°C for 30 minutes prior to application to the gel.

2.17.2 1D-isoelectric focusing gel

1D-IEF was performed using 20x20cm slab gel in a BioRad protein II system. The composition of the gel was 2% NP-40, 4.5% polyacrylamide, 9M urea, and 1% (v/v) ampholyte (Sigma). Before running the sample, wells were rinsed with dH₂O. The bottom reservoir of the IEF apparatus was filled with 20mM H₃PO₄ and top reservoir contained 50mM NaOH. The samples were centrifuged at 8000xg for 4 minutes, and loaded on to the gel. The samples were overlaid with 8-10 μ l of overlay buffer (1:3 dilution of IEF buffer containing 0.1% of bromophenol blue). Gels were run with a voltage gradient of 400V-900V until equilibration (overnight). The gels were then removed and treated with DMSO and DMSO/PPO. The banding patterns were visualised by fluorography by incubating -70°C for 7 days.

2.18 Polymerase chain reaction (PCR)

PCR reactions were carried out in 50 μ l or 100 μ l volumes in sterile 0.5ml tubes. The basic PCR components were as follows: 1xPCR buffer (50mM KCl, 10mM Tris HCl pH 8.3, 1.5mM MgCl₂); 200 μ M of each dNTP (Pharmacia); 25pmoles of each primer; and 2.5 units of *Thermus aquaticus*. (Taq) DNA polymerase (Life Tech) and

DNA template. For certain applications which required the amplification of larger sized PCR products, and also to ensure the accuracy of replication (fidelity), thermostable proof-reading DNA polymerases such as Deep Vent DNA polymerase was used. The reaction buffer (1x) for Deep Vent DNA polymerase (New England Biolabs) contained 10mM KCl, 10mM $(\text{NH}_4)_2\text{SO}_4$, 20mM Tris-HCl (pH 8.8), 2mM MgSO_4 and 0.1% Triton X-100. Deep Vent DNA polymerase was used in combination with Taq DNA polymerase at a 1:10 ratio for extended PCR. These reactions were carried out in Deep Vent PCR buffer. When all the reaction components were assembled, a mineral oil overlay was added and cycling carried out as described, using either a Techne Programmable or Hybaid-Omnigene thermal cycler. Following PCR amplification, 10% of the product was analyzed by gel electrophoresis, as described in section 2.6.1.

The primers used for PCR were synthesized by Oswel DNA services (Dept. of Chemistry, Kings Buildings, Edinburgh) and their sequences are given in appendix. Typically, PCR primers were 18-24 nucleotides in length and approximately 50% GC so that both primers in a reaction had similar annealing temperatures. Primers sequences were selected to avoid 3' end complementarity which would lead to the formation of primer-dimers (Rolf et al. 1992). Primers sequences were checked for specificity for only bovine class I sequences using the GCG program FASTA.

2.18.1 *Polymerase chain reaction for screening of recombinant colonies.*

Recombinant plasmids in bacterial cells were analyzed directly by PCR by the method of Hawker and Billadello (1993). A bacterial colony on a sterile pipette tip or 1 μ l from an overnight culture was used to inoculate 100 μ l sterile distilled water. The sample was boiled for 1 minute and chilled on ice for 5 minutes. 80 μ l was

withdrawn and added to 20 μ l containing: PCR buffer, 1.5mM MgCl₂; 200 μ M deoxyribonucleoside triphosphates (dNTPs); 25pmol of each PCR primer; and 2.5 units of Taq DNA Polymerase (Life Technologies). The samples were subjected to PCR in a DNA Thermal Cycler (Techno). The PCR program used an initial denaturation step consisting of 1 cycle of 94°C for 5 minutes, 55°C for 4 minutes and 72°C for 4 minutes followed by 25 cycles of 94°C for 1 minute, 55°C for 2 min and 72°C for 4 min. After PCR, 15 μ l of each reaction were subjected to electrophoresis on 1.5% agarose gels.

2.18.2 PCR primers

The primers used for the PCR amplification of class I gene fragments and for the construction of expression vector are given in Table 2.2

Primer	Strand	Sequence
770	+	GCTCCCACTSMTGAGGTATT
771	-	CTCGCTCTGGTTRTAGTAGCC
772	-	GATGMAGCATCACTCAGTCCC
773	+	GGCTCTGATGTGTCTCTCACG
2405	-	TCCCSACCCTGACCGGTGTTCTCYTCCCTG
2406	-	GGARGAGAACACCGGTCAGGGTSGGGAGGG
2407	+	GGAGGTCCGGCCGCTTGCCCAGGCTCCCACT CSMTGA
2408	+	GGGGAGACCCGGCCGGACCTCCTC
M0700	+	GGGGCGGCCGTGGGGARGGGCAGCAGTGGG

+ Reads 5'-3'

- Reads 3'-5'

The following ambiguity codes represent mixed-base positions in the primer sequences: S, G or C; M, A or C; Y, C or T; W, A or T; K, G or T; B, G or T or C; D G or A or T; R, A or G.

Chapter 3

PURIFICATION AND CHARACTERISATION OF PHAGE CLONES

3.1 Background

As part of previous work done in our laboratory, fifteen bovine class I genomic clones had been isolated (Hasima, 1992). These clones were isolated from a genomic library prepared in the lambda vector EMBL-3 using peripheral blood mononuclear cell (PBM) DNA from an animal with the BoLA-A10, and A11 serotypes. The 15 clones selected from the library hybridised with both 5'-end and 3'-end probes from the bovine MHC class I cDNA clone pBoLA-1 (Brown et al. 1989), and were considered candidates for full length genes. These clones were transfected into mouse L-cells by calcium phosphate mediated transfection (Hasima, 1992), as described by (Wigler et al. 1979), with the thymidine kinase (tk) gene as selectable marker.

Only one clone, 19.1, showed significant levels of expression of a BoLA class I product. This clone was characterised by restriction mapping, and by serological and biochemical methods. The transfected cell line was shown to express a bovine class I molecule with A11 specificity. The remaining 14 uncharacterised clones potentially represented both the A10 and A11 specificities. One possible reason for the lack of expression in these clones was that all fifteen had been accidentally contaminated with wild type lambda (Hasima, 1992), and the transfected DNA contain only a small proportion of class I DNA from the phage clones. So, the initial part of this project was to plaque purify each of the (class I) phage clones and to transfect the DNA from different phage clones to characterise functional class I products.

3.2 Plaque purification of phage clones

A PCR screening protocol developed by Mason (1992) was used for purification of plaques, instead of conventional methods of screening by hybridisation with specific probes.

3.2.1 *Screening of individual plaques for class I sequences by PCR*

Plaque assays were done on all phage stocks from the 15 clones (1.3, 4.2, 5.2, 6.3, 7.1, 7.2, 7.3, 14.2, 14.3a, 15.2, 17.3x, 17.3y, 18.16, 18.3b and 19.1) isolated by Hasima (1992) as described in section 2.3. Ten individual plaques from each assay were picked for screening of each clone. The single plaques were eluted overnight in 300 μ l of SM (Phage buffer). 100 μ l of eluted phage was precipitated by adding 20 μ l of phage precipitant (20% PEG, 2.5M NaCl) and left overnight at 4°C. Samples were spun at 14000 rpm for 20 minutes and the supernatant was removed carefully. The phage pellets were resuspended in 20 μ l of water and then transferred to PCR tubes.

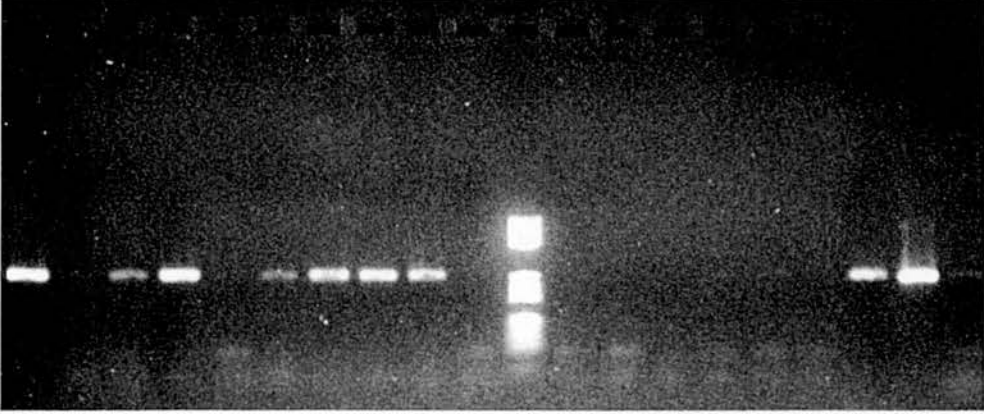
3.2.2 *PCR amplification of class I gene exon 2 and exon 7 sequences*

PCR primers for amplification of exon 2 (770, 771) and exon 7 (773, 772) as given in section 2.18.2 were designed from conserved regions of the published bovine cDNA sequences (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Garber et al 1993). The PCR mixture was assembled as described in section 2.18. Cycle conditions for PCR amplification were: denaturation for 90 sec at 94°C followed by 25 cycles of 30s at 94°C, 30s at 55°C and 30s at 72°C. The final extension time was 2 minutes 72°C. Following exon 2-specific PCR amplification, plaques with a class I insert gave a product of 270 bases (Fig 3.1). Positive plaques were further screened using exon 7 primers to give a product of 200 bases (Fig 3.2).

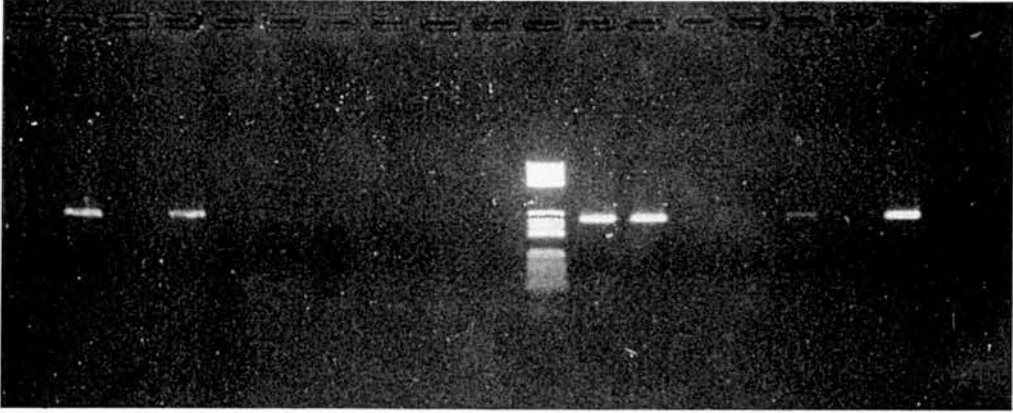
Fig. 3.1 PCR amplification of the 270bp exon 2 fragment from single phage plaques containing class I genes, analysed by gel electrophoresis. Lanes 1 to 10 represent the amplification products from single plaques of phage clone 15.2. Lanes 11 to 19 are single plaque amplifications from clone 1.3.

Fig. 3.2 PCR amplification of the 200bp fragment from exon 7 to 8 from individual plaques analysed by gel electrophoresis. Lanes 1 to 10 represent plaques from clone 4.2 and lanes 11 to 19 are plaques from clone 17.3x.

1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19



1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15 16 17 18 19



Despite high degree of contamination, 2-3 class I -positives plaques were identified from each clone. However, no positives plaques were found from clone 7.1.

3.2.3 *Selection of different clones from exon 2 sequences*

Once the various phage clones were purified, the next stage was to determine how many different genes were represented by the 15 clones. The quickest way to differentiate the clones was to directly sequence the PCR-amplified polymorphic exon 2 by cycle sequencing as described in section 2.8.3. This avoided the need to subclone the PCR products for sequencing. Cycle sequencing of PCR amplified exon 2 fragments gave a high background so that very long reads from the gels were not possible. However, the small area of exon 2 sequence which was readable was sufficient to show differences among the phage clones (Fig 3.3). When the exon 2 sequences from the different clones were compared, at least three different sequences were found. The three groups which shared readable exon 2 sequence are summarised in Table 3.1.

Group 1	Group 2	Group 3
19.1	4.2	1.3
5.2	14.2	
6.38	14.3a	
7.24	15.2	
7.3	17.3x	
17.3y		
18.16		
8.3b		

Table 3.1: Three groups of phage clones which were similar in exon 2 sequence.

	10	20	30	40	50	60	70	75
16	AGCCCCGCTTCATCGCCGTCGGCTACGTGGACGACACGCAGTTCACACGGTTCGACAGCGACGCCCCGAATCCAA							
9	-----							
3	-----							
4	-----							
1	-----							
16	-----							
3B	-----							
3Y	-----							
1	-----							
5	-----							
21	-----							
22	-----							
3A	-----							
3X	-----							

3.3 Alignment of exon 2 sequences from the fourteen genomic class I clones, obtained by the sequencing of the exon 2 PCR product. Dashes (-) indicate homology with the 19.1 sequence whereas dots (.) indicates unbreakable areas of the sequence. The second decimal digit of clone indicates the plaque number.

Further, when comparative gels were run with A, G, C and T lanes from different clones side by side visible differences were seen between the apparently identical 4.2 and 15.2 clones in regions of the gel from which clear sequence could not be read (Fig 3.4).

3.3 Amplification of class I genes from genomic clones

Six lambda clones (1.3, 4.2, 15.2, 17.3x, 18.3b and 19.1) were selected for further study based on exon 2 differences. Although the clone 18.3b looked similar to 19.1 in comparative runs of A, G, C and T, due to lack of exon 2 sequence for comparison it was included in this experiment for further analysis. To ease the characterisation of the clones, a fragment spanning the exon 2-exon 8 fragment was amplified from phage DNA using 5'-end forward primer for exon 2 (770) and the 3'-end reverse primer for exon 8 (772) as described earlier. PCR conditions used were slightly modified for amplification of the larger sized fragments: 90s at 94°C for denaturation, followed by 30 cycles of 30s at 94°C, 30s at 60°C and 2 minutes at 72°C with the final extension time 5 minutes at 72°C. The amount of Taq DNA polymerase was also increased from 1 unit to 2.5 units per reaction. All six clones selected for further characterisation gave a product of 3.2kb using the above primers (Fig 3.5a).

3.4 Characterisation of the exon 2-exon 8 fragments

The 3.2kb fragment amplified from the different clones were digested with six restriction enzymes in attempts to further characterise the different clones. Aliquots of 5 μ l of the amplified product were digested with the restriction enzymes *Sma* I, *Sac* I, and *Rsa* I for 3 hours using protocol recommended by the manufacturer. The digestion products were resolved on 1% agarose for *Sac* I and *Sma* I or 6% Acrylamide gels for *Rsa* I as shown in (Fig 3.5b and 3.5c).

Fig. 3.4 Comparative sequence analysis of purified class I exon 2 PCR products. Clone names are given above each set of A, G, C, T sequence track.

A

C

G

T

4.2
15.2
1.3
19.1
18.3b
17.3x
4.2
15.2
1.3
19.1
18.3b
17.3x
4.2
15.2
1.3
19.1
18.3b
17.3x

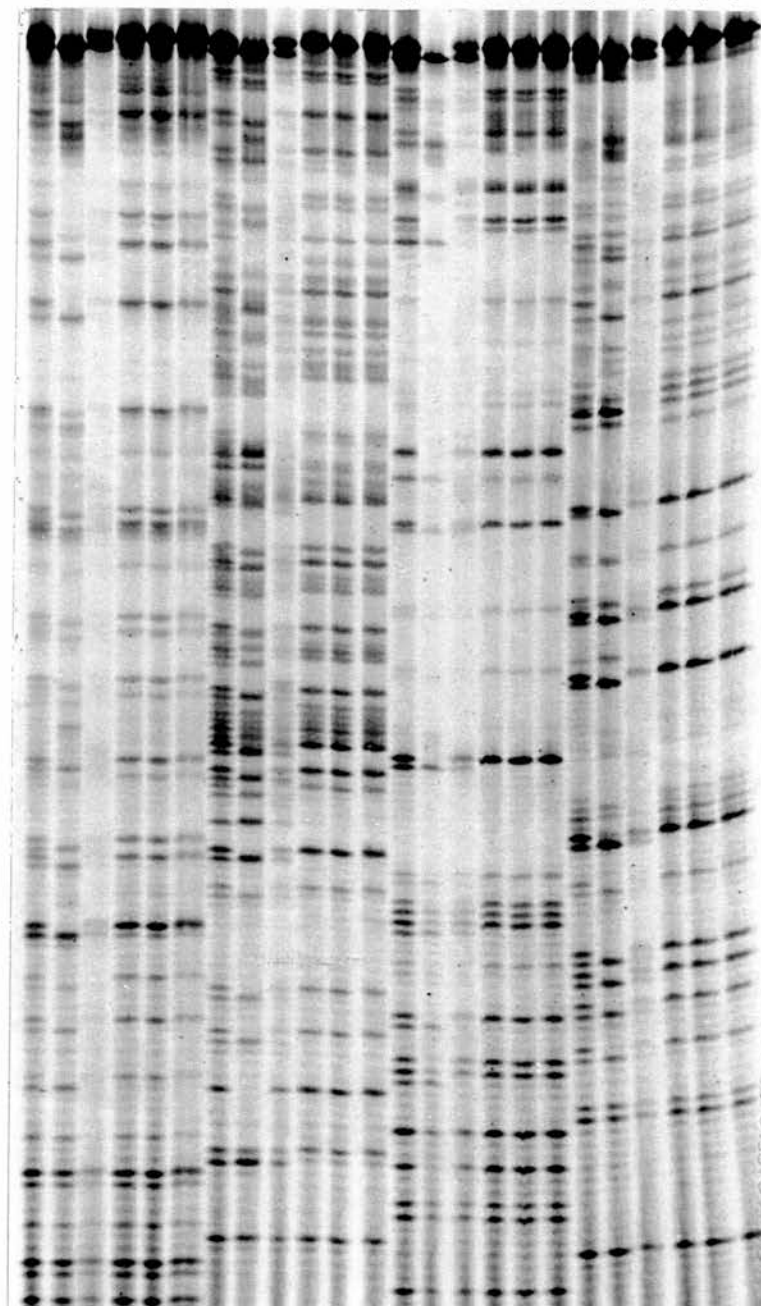


Fig. 3.5a PCR amplification of a 3.2kb exon 2 - exon 8 fragment from the six phage clones (Lane 2 to Lane 7) using primers 770 and 772. 10% of each PCR product was analysed by gel electrophoresis on a 0.75% agarose gel. The molecular weight markers (1kb ladder) are shown in lane 1.

Lane 2: clone 1.3

Lane 3: clone 4.2

Lane 4: clone 15.2

Lane 5: clone 17.3x

Lane 6: clone 18.3b

Lane 7: clone 19.1

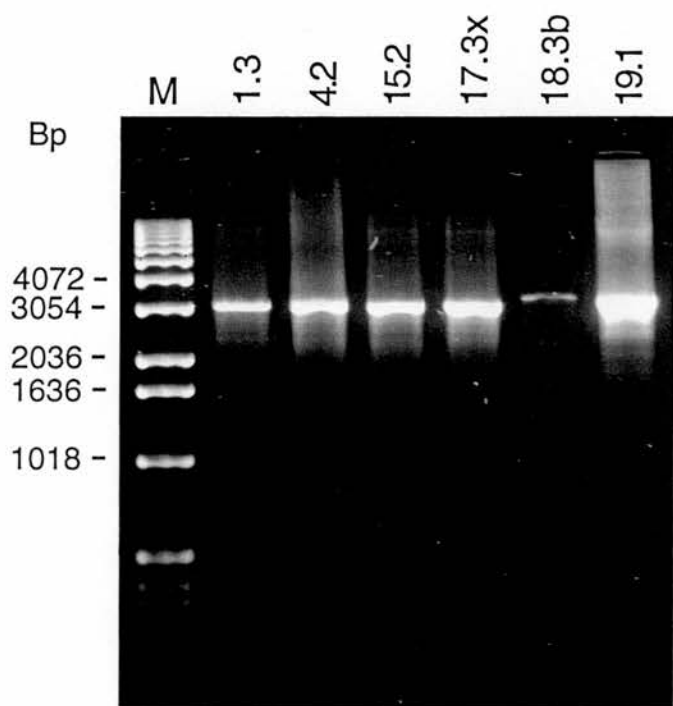
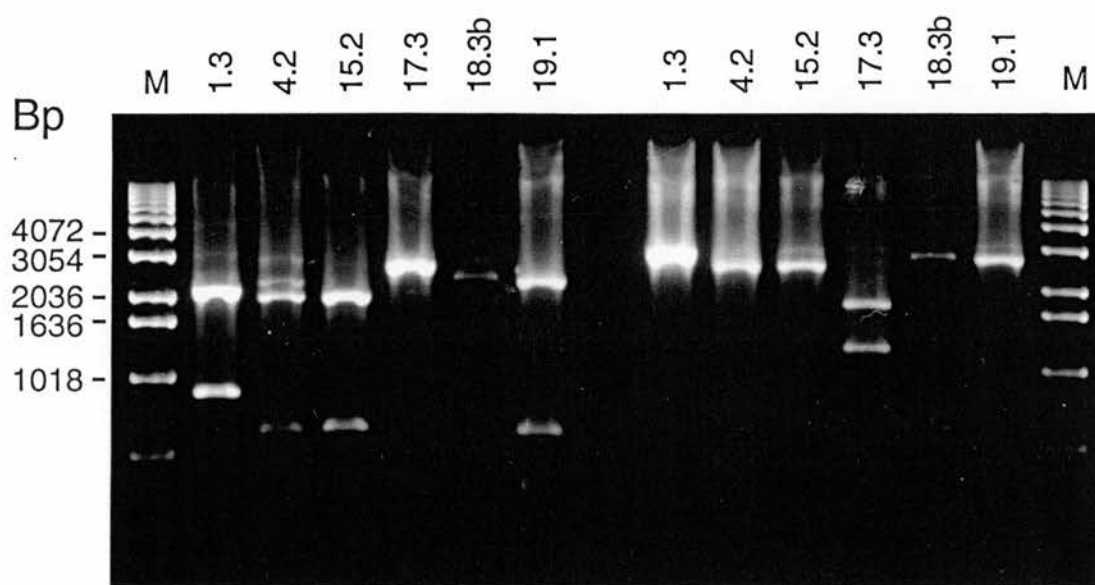


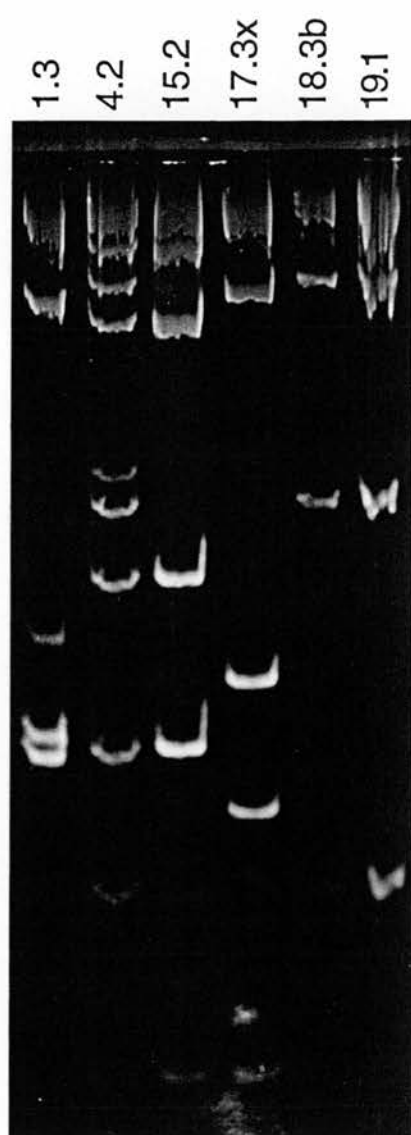
Fig. 3.5 b and c Restriction digests of the 3.2kb amplified PCR fragment from phage clones 1.3, 4.2, 15.2, 17.3x, 18.3b and 19.1 with *Sma* I, *Sac* I and *Rsa* I. *Sma* I and *Sac* I digests were analysed on 0.75% agarose gel. *Rsa* I digests were run 5% acrylamide gel. Molecular weight marker sizes are included in the picture.

Sma1

Sac1



Rsa1



In the *Sma* I digests clones 1.3, 4.2 and 15.2 shared a band of 2.1kb (Fig 3.5b). However, clone 1.3 had another band of 900bp while in clones 4.2 and 15.2 this band was split into two bands of 600bp and 300bp. Both 19.1 and 18.3b shared bands of 600bp and 300bp with top band of a smaller size compared to 4.2 and 15.2. In *Sac* I digestion four clones (4.2, 15.2, 18.3b and 19.1) had similar restriction patterns, 1.3 did not cut and 17.3x showed a different pattern from all of other clones. All of the clones generated different restriction pattern with *Rsa* I except 18.3b which had a 19.1- like pattern. These results indicated that five clones (1.3, 4.2, 15.2, 17.3x and 19.1) were different patterns with one or more enzymes. Clones 19.1 and 18.3b were found to have similar restriction patterns with all of the enzymes used, so 18.3b was not included in above group for further analysis.

3.5 Discussion

In order to screen plaques for class I genes, primers were designed specifically for exon 2 and the exon 8/3'-untranslated region. Primers sites were chosen based on regions conserved in all of the published BoLA cDNA sequences (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991 and Garber et al. 1993), thus maximizing the chance of amplifying class I sequences in genomic clones. The primers were located at the furthest ends of the genes for which complete sequence data were available, and plaques which gave a positive amplification product with both sets of primers gave further evidence of the completeness of the clones. The five clones chosen for further study all gave a product of 3.2kb on amplification of the class I exon 2-exon 8 fragment. This fragment size is in accord with the published data, as the size of class I genes in swine, mouse and man range from 3.6kb to 5kb (Satz et al. 1985; Strachan et al. 1984; Weiss et al. 1985 and Isamat et al. 1990). Furthermore, digestion of the 3.2kb PCR fragments from these genomic clones with six selected

restriction enzymes generated unique restriction patterns for each clone. There is growing evidence that more than one loci are expressed in cattle (Garber et al. 1993; 1994; Al-Murrani et al. 1994). In order to investigate the expression and function of the individual class I products encoded by these clones, purified phage DNA was transfected into mouse Ltk⁻ cells. These experiments are discussed in subsequent chapters.

Chapter 4

TRANSFECTION OF BOVINE CLASS I GENOMIC CLONES

4.1 Introduction

Transfection of cloned genes encoding products with immunological relevance, such as major histocompatibility antigens has provided important information on the structure, function and gene regulation of these molecules. This experimental system has been used to identify functional MHC class I molecules of swine, murine, ovine and human origin in mouse L cells (Singer et al. 1982; Goodenow et al. 1982; Evans et al. 1982a; Lemonnier et al. 1982). In the bovine genome there is evidence for a large number of class I genes (at least 10) from southern blot analysis using a human class I cDNA probe (Lindberg and Andersson, 1988). In contrast, the use of bovine allo-antisera in a lymphocyte micro-cytotoxicity assay (Spooner et al. 1979a) suggests that a single highly polymorphic class I product is expressed by the BoLA system. The actual number of expressed MHC class I genes in cattle is as yet unknown. Biochemical and cDNA cloning experiments, however, suggest the presence of two or three expressed class I genes in cattle (Ennis et al. 1988; Bensaid et al. 1991; Ellis et al. 1992; Al-Murrani et al. 1993, 1994; Garber et al. 1993, 1994). Because of the lack of availability of amino acid sequence data corresponding to each of the serologically defined BoLA class I specificities, the identification of genes encoding each of the corresponding molecules has to rely on transfection experiments. Shotgun transfection and expression of bovine class I molecules has been described previously (Toye et al. 1990), but the genes involved were not isolated. In order to investigate the expression and function of individual class I locus products, transfection and characterisation of a series of BoLA class I genomic clones isolated from a single animal was performed. The selection of the class I clones described in this chapter, and the transfection of one clone (19.1), was the

subject of a previous study (Hasima, 1992).

The murine L cell has been the most commonly used recipient for studies on the function and expression of transfected genes encoding murine and human class I antigens. Most, but not all anti-HLA monomorphic and polymorphic monoclonal antibodies detect HLA/mouse heavy chain β_2m hybrid molecules (Malissen, 1986). It has been shown that heterologous association between mouse β_2m and HLA class I heavy chain is sufficient for cell surface expression (Brodsky and Parham, 1982). Various workers have also shown that free β_2m can exchange with β_2m associated with heavy chains expressed on the cell surface (Kefford et al. 1984; Bernabeu et al. 1984). However, the failure of expression of HLA-B27 at the L cell surface after transfection was due to the inability of the HLA-B27 gene product to interact with murine β_2m (Rein et al. 1987). When the HLA-B27 gene was transfected into L cells containing the human β_2m gene, expression was detected. The production of L cell transfectants, and *in vitro* mutagenesis of class I genes provide important tools in location of determinants on class I molecules which are recognised by antibodies and cytotoxic T cells.

4.2 Transfection of class I-positive phage clones

A wide of variety of transfection techniques have been described:

- i) Polycations (Kawai and Nishizawa, 1984);
- ii) Calcium phosphate (Graham and Van der Eb, 1973);
- iii) Liposome fusion (Cudd and Nicolau, 1984);
- iv) Addition of DNA via electroporation (Neumann et al. 1982);
- v) Transfection with viral vectors (Sudgen et al. 1985);
- vi) Micro-injection (Capecchi et al. 1980);

However, all of these methods suffer from one or more problems related to either cellular cytotoxicity, inconvenience or inefficiency of DNA delivery. During this project we used a cationic lipid (DOTAP) that forms liposomes which interact with tissue culture cells to facilitate delivery of functional DNA. Liposomes are relatively non-toxic, and yield 5-100 fold higher transfection efficiencies than either calcium phosphate or DEAE dextran methods (Felgner et al. 1987). Phage DNA, prepared by CsCl step gradient purification (section 2.3) from the five clones 1.3, 4.2, 15.2, 17.3x and 19.1, was transfected in mouse Ltk⁻ cells as described in section 2.13.

4.2.1 Optimization of relative dose of phage DNA and pTK marker

Since the total quantity of DNA transfected in the DOTAP method is 5 μ g, as per manufacturer's instructions (section 2.13), a preliminary experiment was designed to optimize the dose of clone DNA and marker (pTK) for the maximum level of class I gene expression. This was done using the phage clone 19.1 which had previously shown to express BoLA-A11 specificity on transfection.

Four different combinations of 19.1 phage DNA and marker pTK were used as follows:

19.1 (4 μ g) + pTK (1 μ g); 19.1(3 μ g) + pTK (2 μ g); 19.1 (2 μ g) + pTK (3 μ g); and 19.1 (1 μ g) + pTK (4 μ g). The transfections were carried using the protocol described in section 2.13.

Results of the dose titration are summarised in Table 4.1 :

19.1 DNA	pTK	Number of HAT resistant colonies	Expression of clone 19.1 (%)
1 μ g	4 μ g	> 1000	2.8
2 μ g	3 μ g	> 750	5
3 μ g	2 μ g	> 400	6.2
4 μ g	1 μ g	> 125	17.8

Table 4.1 Transfection efficiency and class I expression of different doses of clone 19.1 and pTK.

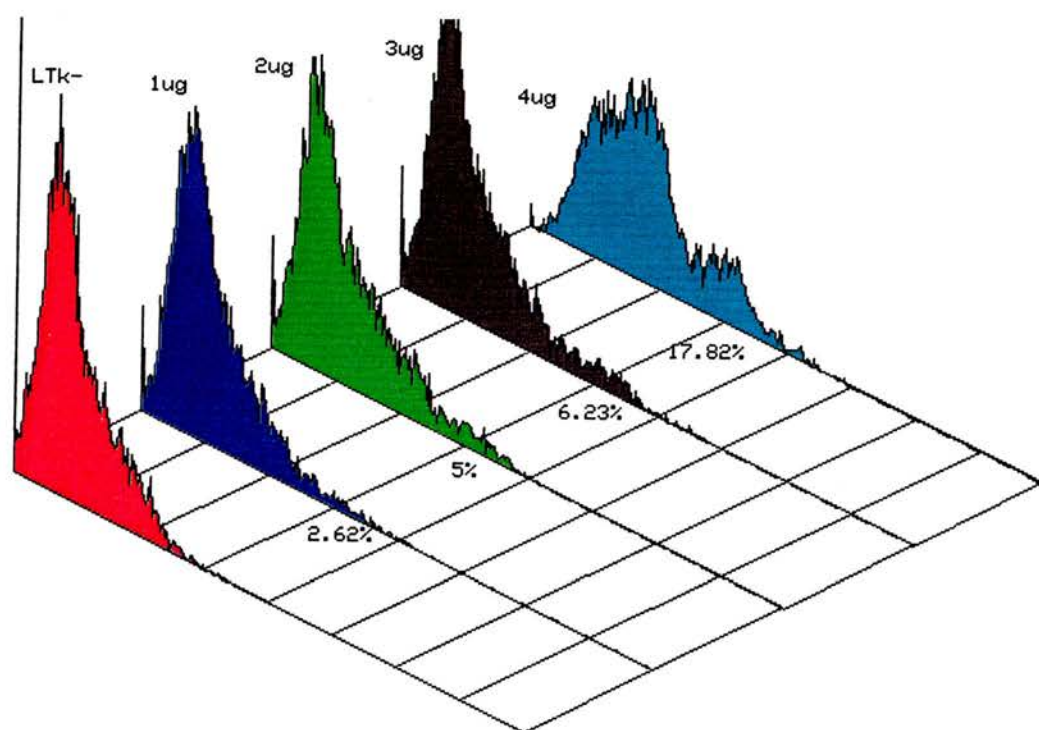
The results illustrated in the table indicate that the maximum level of class I product expression (17.8%) for clone 19.1 was obtained when 4 μ g of 19.1 phage DNA and 1 μ g of pTK was used for transfection (Fig 4.1). Interestingly, the number of HAT resistant colonies was lowest at this phage: pTK ratio. All the transfection experiments carried out during this project were done using this optimised ratio of 4 μ g of class I-carrying phage or plasmid DNA to 1 μ g of pTK marker.

4.2.2 Analysis of transfected mouse L cell lines

The phage clones (1.3, 4.2, 15.2, 17.3x and 19.1) characterised by their exon 2 sequences and by restriction analysis of the amplified 3.2kb class I gene fragment, were transfected into mouse L cells, using the DOTAP protocol with the optimum ratio of 4 μ g phage DNA to 1 μ g pTK. After 2 to 3 weeks, HAT resistant colonies were analysed for BoLA class I expression by using class I-specific monoclonal

Fig. 4.1 Histogram Fluorescence-activated cell scan (FACS) analysis of bovine class I molecules on the surface of L cells transfected with different relative doses of 19.1 phage DNA and pTK marker. The cells were labelled with murine monoclonal antibody specific for a non-polymorphic determinant on bovine MHC class I products (IL-A88). Different amounts of phage DNA transfected are shown by different colours.

Red : Ltk; Royal Blue : $1\mu\text{g}$; Green : $2\mu\text{g}$; Black : $3\mu\text{g}$; Turquoise Blue : $4\mu\text{g}$.



antibodies: Anti HLA mAb W6/32 and anti BoLA class I mAb IL-A88 in flow cytometry section 2.14. Neither W6/32 nor IL-A88 cross-reacted in this test with murine class I antigens expressed by the L cells. Of the five clones transfected, only 19.1 and 4.2 showed expression at the L-cell surface. The phage clone 19.1 which had been previously shown to express a bovine class I molecule with A11 specificity (Hasima, 1992) served as a control in this experiment. Clone 4.2 showed 7.6% of cells expressing bovine class I molecules on their cell surface when subjected to FACScan analysis with IL-A88 (Fig 4.2), while clone 19.1 showed 17.8% cells positive for class I expression in (Fig 4.1). The other three clones (1.3, 15.2 and 17.3x) did not show any significant class I expression. The cells transfected by these three clones were nevertheless sorted by MACS in case a very small population of class I expressing cells could be isolated. However, no class I expression was detected even after MACS sorting of these cells. The 19.1 and 4.2 transfected cell lines were enriched over five rounds of sorting and expansion in culture to increase the proportion of cells expressing class I up to 80% (Fig 4.2). In addition, the transfectant were routinely MACS sorted so that most experiments were done with a expression level of between 70 and 80%.

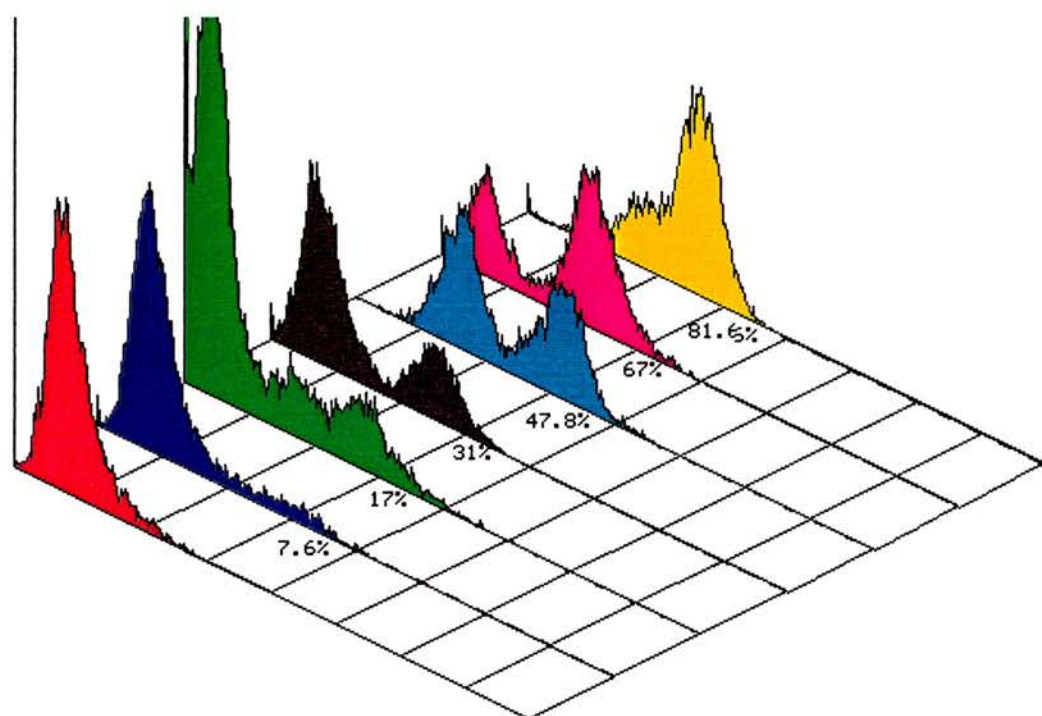
4.3 Characterisation of the clone 4.2 transfected cell line

The bovine class I product expressed on the 4.2 transfected cells was characterised by flow cytometry analysis, BoLA class I typing by a micro-lymphocytotoxicity assay (Spooner et al. 1979a), T cell cytotoxicity assays (Teale et al. 1985) and iso-electric focusing (Joosten et al. 1988). The 19.1 transfectant cell line had been previously characterised by all of these methods and had been shown to express an A11-specific BoLA class I product. Therefore the 19.1 transfected cell line was used as a control in the characterisation of the 4.2 expressed product.

Fig. 4.2 Histogram FACS analysis of progressive sorting for class I expression of mouse Ltk⁻ cells transfected with DNA from the 4.2 phage.

The figure show the relative fluorescence of transfected cell populations stained with IL-A88 at different stages of sorting, represented by different coloured peaks in the histogram.

Red : pTk; Royal Blue : Primary transfection; Green : First sort; Black : Second sort; Turquoise Blue : Third sort; Pink : Fourth sort; and Yellow : Fifth sort.



in the characterisation of the 4.2 expressed product.

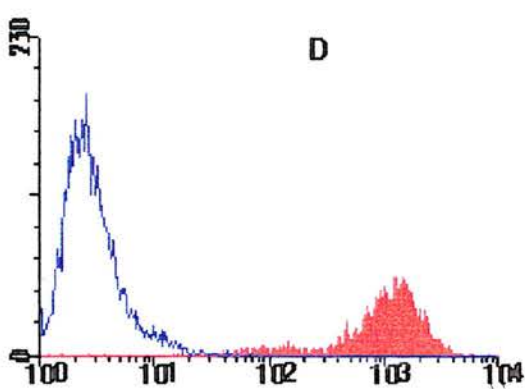
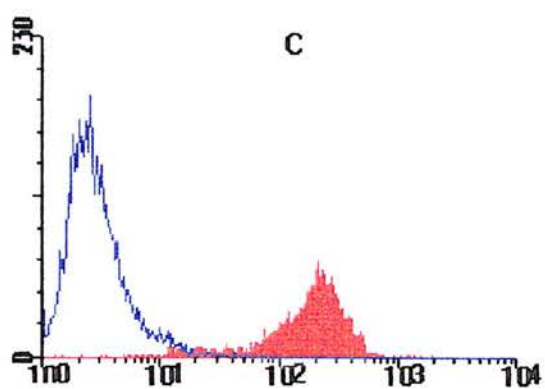
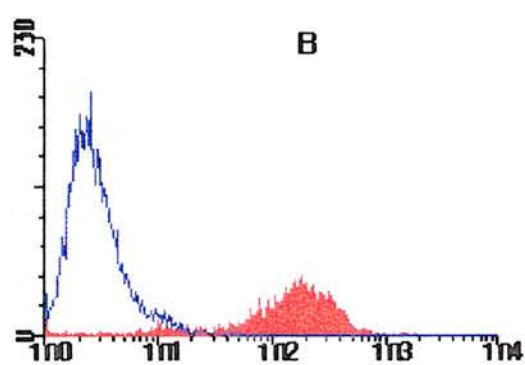
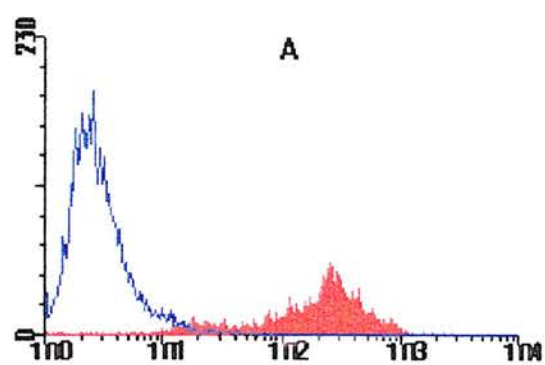
4.3.1 Screening of the 4.2 transfected cell line with BoLA A10-specific monoclonal antibodies

Since mAbs for one of the haplotypes on the cloned animal were available, the 4.2 transfected cell line was first analysed using a group of A10-specific mAbs. Three A10-specific mAbs (IL-A7, IL-A10 and IL-A34; Toye et al. 1990) were used in flow cytometry experiments to determine whether the product expressed by the 4.2 gene had A10 specificity. All three mAbs recognised A10 serotype PBM (Fig 4.3), but failed to recognise the product expressed on 4.2 transfected cells.

4.3.2 Micro-lymphocytotoxicity assay

To identify the expressed product on the 4.2 transfectant, the micro-lymphocytotoxicity assay described in section 2.16 was used with specific allo-antisera routinely used to define the A10 and A11 specificities (Davies et al. 1994). The 4.2 transfected cells were lysed with three of the four A11 allo-antisera used (Table 4.2). Control L cells were not killed with any of the A11 sera, and neither control nor transfected L cells were killed with sera defining the A10 specificity. The 19.1 transfected cell line was lysed with all four A11-specific allo-antisera. The killing of the transfected cell lines was observed at higher concentrations of allo-antisera than normal cattle PBM. The results are summarised in Table 4.2.

Fig. 4.3 FACS analysis of PBM from animal 10769 (BoLA-A10, A11) (A) with BoLA-A10 specific mAb IL-A7, (B) the BoLA-A10 specific mAb IL-A10, (C) the BoLA-A10 specific mAb IL-A34 and (D) the pan-class I mAb IL-A88.



	A11				A10	
Cells	Ed 73	Ed 76	Ed 102	Ed 110	Ed 69	Ed 71
PBM	+	+	+	+	+	+
Ltk ⁻	-	-	-	-	-	-
Ltk ⁻ 4.2	+	+	-	+	-	-
Ltk ⁻ 19.1	+	+	+	+	-	-

Table 4.2: Results of micro-lymphocytotoxicity assay

4.3.3 *T cell cytotoxicity assay*

A close correlation has been established between serologically defined BoLA specificities and the activity of allospecific T cells (Teale et al. 1985; Spooner et al. 1987). Functional analysis of the product expressed on clone 4.2 transfected cells was performed using anti-A11 and anti-A10 allo-reactive cytotoxic T lymphocytes (CTL) as described in section 2.12. In this assay the 19.1 transfected cell line which expressed A11 specificity was used as a control. The A11-specific CTL line killed PBM with A11 specificity and both the 19.1 and 4.2 transfected cell lines in the same manner (Fig 4.4), whereas neither the 19.1 or 4.2 transfected cell lines were lysed by the A10-specific CTL line. The control L cells were not killed by either CTL line. This result further supported the findings that both the 19.1 and 4.2 gene products expressed A11 specificity.

Fig. 4.4 BoLA class I-specific ^{51}Cr -release cytotoxicity assay of clone 19.1 and 4.2 - transfected cells with **A)** anti A-11 specific and **B)** anti-A10 specific CTL. The panel of target cells as follow:

- o *T.annulata* from the donor animal, 10769, BoLA A10/A11

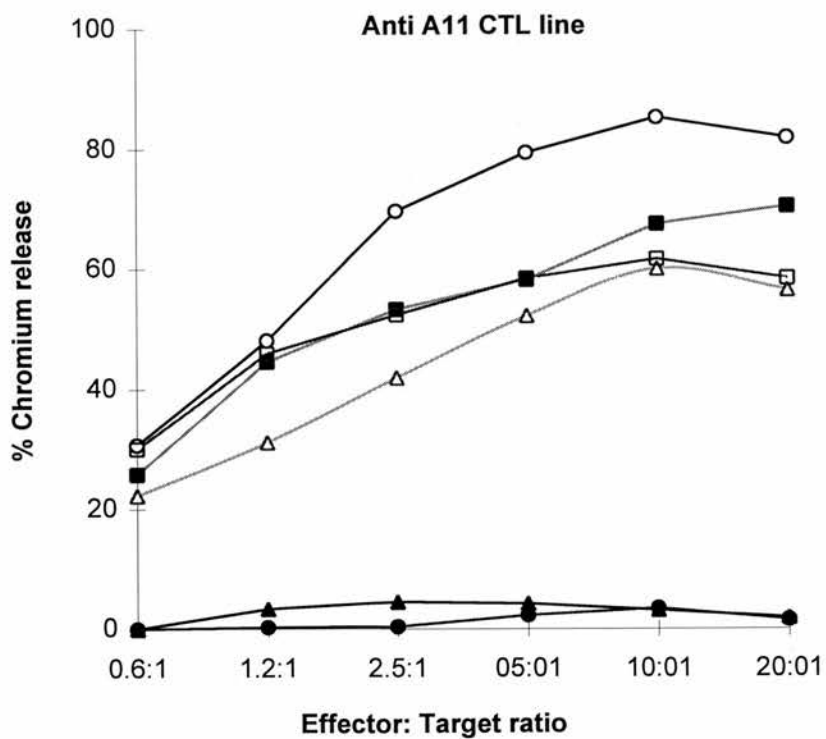
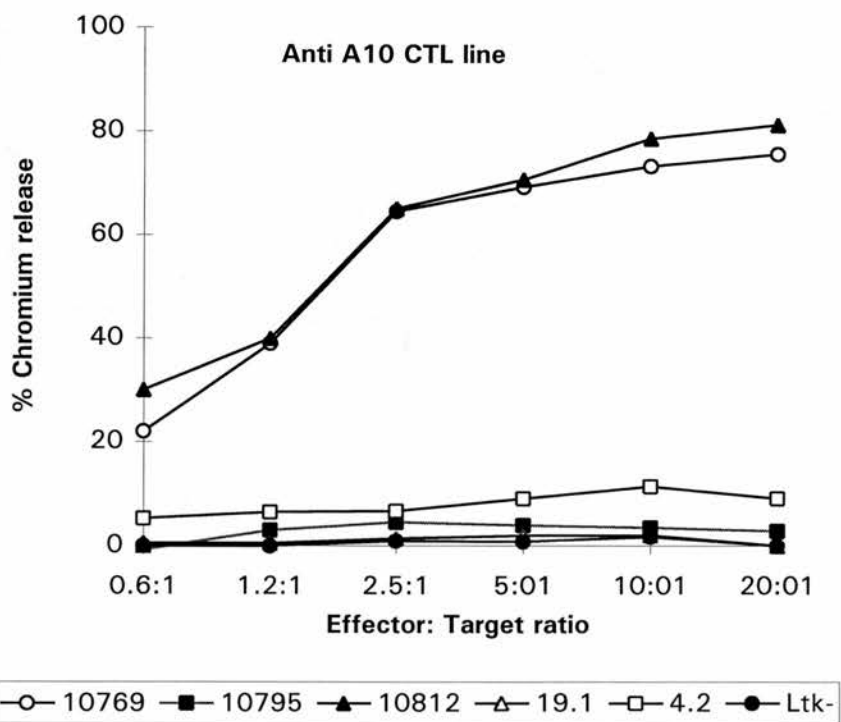
- 10795 PBM, BoLA A11/20

- ▲ 10812 PBM, BoLA A10/A31

- △ Clone 19.1 transfected L-cells

- Clone 4.2 transfected L-cells

- Control L cells.



4.3.4 *Isoelectric focusing of the clone 4.2 gene product*

W6/32 (IgG2a) was used for immuno-precipitation of BoLA molecules from 19.1 and 4.2 transfected cells, and from A11 PBM, as previously described (Joosten et al. 1988). W6/32 recognises a conserved epitope present on BoLA molecules (Brodsky et al. 1981), which is dependent upon the association of β_2m (Parham et al. 1979; Jefferies and MacPherson, 1987). The banding pattern of the PBM contained several charge variants (Fig 4.5) as described earlier (Al-Murrani et al. 1994), a number of which are associated with the A11 haplotype. Both the 19.1 and 4.2 transfectants showed a single band which had the same iso-electric point and corresponded with one A11-specific band observed on PBM, which was not seen in the control Ltk⁻ cells (Fig 4.5). The difference in the intensity of the band derived from the transfected cells and PBM may be due to variation in the level of the expression of transfected gene. By all of the above methods the 4.2 expressed product could not to be discriminated from the 19.1 product. Although with 1D-IEF it was expected to see a difference in the mobility of the 19.1 and 4.2 transfectant bands, as both these genes had different sequences in exon 2. These results required further investigation of the transfected cell lines to ensure that they contained the proper DNA.

4.3.5 *PCR amplification of exon 2 from genomic DNA of the 4.2 and 19.1 transfected cell lines*

In order to determine whether the 19.1 and 4.2 transfected cell lines contained the proper DNA, PCR amplification of exon 2 was performed on genomic DNA extracted from the 19.1 and 4.2 transfected cell lines as described in section 2.7 and 3.3. The exon 2 PCR products were TA subcloned and four TA clones from each transfectant were assayed for the presence of the diagnostic *Kpn* I site which was

Fig. 4.5 Isoelectric focusing of class I antigens. The gel shows ^{35}S -labelled molecules immuno-precipitated with anti-HLA class I monoclonal antibody W6/32 which recognises a monomorphic determinant on class I heavy chain associated with $\beta_2\text{m}$.

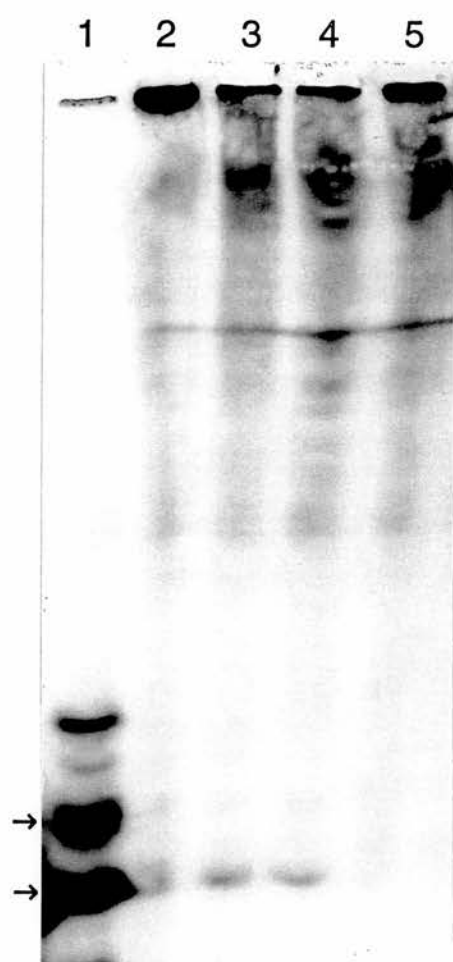
Lane 1 : PBM from animal with BoLA-A10/A11 class I specificity. Arrow indicates A11 specific bands.

Lane 2 : L cells transfected with 19.1 phage DNA.

Lane 3 : L cells transfected with 19.1 reconstructed plasmid pBoLA-25.

Lane 4 : L cells transfected with 4.2 phage DNA.

Lane 5 : Ltk⁻ control, untransfected cells.



known to be present in exon 2 sequence of clone 4.2 but not 19.1 (Fig 3.3). All four TA clones from the 4.2 transfectant could be digested with *Kpn* I to give fragments of 204 and 66bp from exon 2, but the fragment amplified from the 19.1 transfected cells did not cut with *Kpn* I (Fig 4.6). Furthermore, partial sequencing of the exon 2 TA clones demonstrated that the 19.1 exon 2 clones looked like 19.1 and the 4.2 exon 2 clones looked like the 4.2 phage sequence.

4.4 Discussion

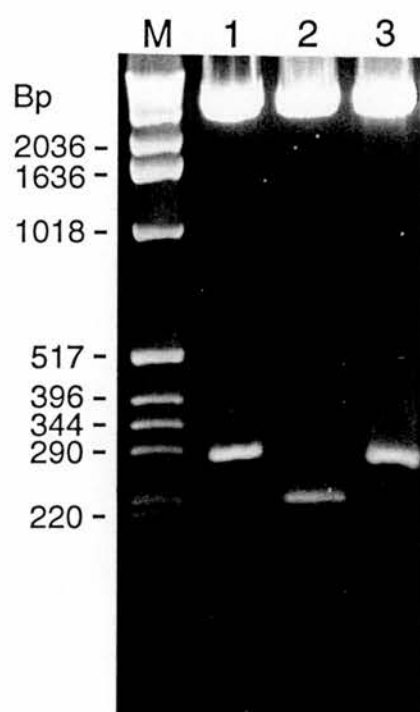
The cell surface expression of BoLA class I molecules by transfected cell lines was revealed by flow cytometry analysis with IL-A88. Micro-lymphocytotoxicity results showed that both the 19.1 and 4.2 transfected cell lines expressed BoLA-A11 specificity. However, lysis of the transfected cell lines was observed either at undiluted or 1:2 dilution of allo-antisera and with a significantly longer incubation than PBM, suggesting that the degree of expression of the transfected gene was much reduced, or that L cells are more robust than cattle PBM. Three A11-specific allo-antisera used in the micro-lymphocytotoxicity assay lysed the 4.2 transfected cells, but the 19.1 transfected cells were lysed with all four anti-A11 allo-antisera. In previous experiments the Ed76 allo-antiserum was found not to lyse the 19.1 transfected cell line (Sawhney et al. 1995). These variations in results both within and between the transfected cell lines could be due to variations between stocks of antisera, or might reflect variable BoLA class I product expression on the transfected cells. Complement mediated lysis of uncloned populations of human transformed cells transfected with HLA-A3 and HLA-Cw3 genes has also been shown to give varying results (Lemmonier et al. 1982), while consistent lysis was seen when cloned population of transfected cells selected for high expression were used. Reduced levels of expression was also observed on the transfectants at confluence.

Fig. 4.6 *Kpn* I restriction digestion of exon 2 270bp fragment amplified from genomic DNA prepared from the 19.1 and 4.2 transfected cell lines.

Lane 1: Uncut PCR amplified exon 2 from the 4.2 transfected cell line.

Lane 2 : *Kpn* I digest of exon 2 amplified from the 4.2 transfected cells.

Lane 3 : *Kpn* I digest of exon 2 amplified from the 19.1 transfected cells.



Therefore, the growth stage of the transfected cells may also have had an influence on the results of some of these assays.

The products from the 19.1 and 4.2 genes were indistinguishable both serologically and by CTL assays, and were thought to be components of the A11 haplotype. Several workers have used 1D-IEF to characterise serologically defined BoLA class I haplotypes, and BoLA-A locus polymorphisms (splits) that were not resolved using the available serological reagents. Interestingly, the bands from the 4.2 and 19.1 transfected cell lines appeared identical on IEF, but the cell lines clearly carried BoLA genes with different exon 2 sequences as evident from previous results. Many HLA antigens such as A3.2 and B7.2; B8 and B27.5; B13 and Bw47 have similar pIs and so have been found difficult to discriminate using IEF (Guttridge and Klouda, 1989). These observations support the view that 4.2 and 19.1 encode distinct elements of the A11 haplotype, and so probably represent different class I loci. To study this possibility further, the structural organisation and DNA sequence of the 4.2 gene was determined. The coding region (exon 2 -exon 8) from clone 4.2 was PCR-amplified from phage DNA, and was subcloned into pBoLA-21 for sequencing (chapter 7). These results and sequence comparison of the 4.2 gene with the 19.1 gene are discussed in chapter 8.

Three clones did not express on transfection (1.3, 15.2 and 17.3x). There are several possible reasons for this. Firstly, these clones might contain truncated class I genes, ie without functional promoter sequences or other elements necessary for transcription and translation. The completeness of all of the clones was determined by hybridisation using the cDNA clone pBoLA-1, which is truncated at both 5'- and 3'-ends, so that the lack of some 5' or 3' sequence would not have been

detected. In addition, the PCR experiments done in chapter 3 to characterise these clones used primers in exon 2 and exon 8, so that these did not give any further information regarding the completeness of genes or of the presence of functional class I promoters. Alternatively, these clones may carry pseudogenes which could not be expressed, or non-classical class I genes whose expression is tissue-specific or whose products could not be detected because of lack of appropriate serological reagents. In addition, there are only a few anti-BoLA class I mAbs available, and it is not clear whether IL-A88 recognises the products of all class I loci. The other monomorphic anti-BoLA class I mAb, IL-A19, could not be used in these experiments as it was found to cross-react with murine class I products. To try to distinguish between these possibilities, the coding region of clone 19.1 was replaced with the corresponding gene fragment from the non-expressing clones. These results of these experiments are described in chapter 6.

Chapter 5

SUBCLONING AND SEQUENCING OF 19.1 GENE

5.1 Introduction

DNA sequencing studies of a number of mammalian genes have examined the molecular basis of genetic diversity. Most studies have focused on genes from different mammalian species or, within a species, have compared non allelic genes from multigene families. Much less information is available on nucleotide diversity between allelic genes. It is of particular interest to examine the molecular diversity of the MHC class I gene family because polymorphism is related to the function of the MHC-encoded molecules. The structural diversity in mouse and human class I alleles (Parham et al. 1989; Pease et al. 1991) is found mostly in the amino terminal domain of the glycoproteins, localised within the peptide binding cleft. It appears that this diversity has been selectively maintained because of its influence on the spectrum of peptides that can bind and be presented to T cells. The class I proteins have domains with structure similar to those noted among various members of immunoglobulin gene superfamily. The domain structure of these proteins is also reflected in the molecular organisation of the corresponding class I genes with a separate exon encoding each domain. The characteristic features of functional class I genes expressed at the cell surface are the leader peptide (exon 1), three extracellular domains (α_1 , α_2 and α_3) approximately of 90 amino acids each, a hydrophobic transmembrane domain which anchors the molecule in the plasma membrane (exon 5) and the cytoplasmic domain which is formed by exon 6- exon 8 and contains a conserved site for serine phosphorylation. The α_1 and α_2 domains form antigen binding site and are highly polymorphic, α_3 is relatively conserved and is the binding site for the non-MHC- encoded component β_2 -microglobulin (β_2m). This domain displays amino acid homology with the constant region domains of

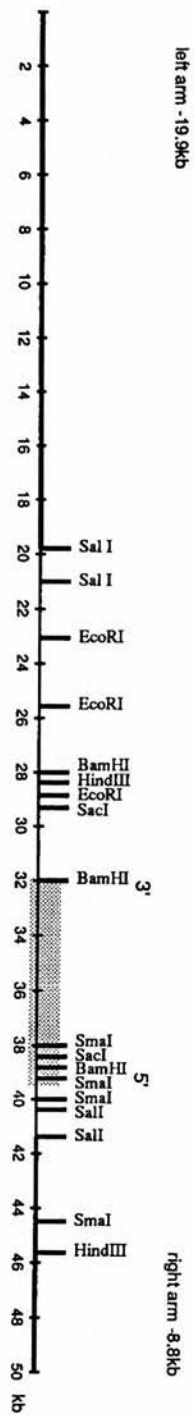
immunoglobulin molecules. The transmembrane and cytoplasmic domains are generally well conserved, but may contain locus-specific substitutions in various class I molecules. The lambda clone 19.1, isolated from a genomic library, expressed a BoLA-A11 specificity on transfection. In order to correlate this serological specificity with the structural organisation of the genomic clone, the 19.1 gene was subcloned into plasmid pBR322 to obtain sequence information.

5.2 Subcloning of the 19.1 gene in pBR322

The lambda EMBL3 clone 19.1 contained a 21kb DNA insert with all of the pBoLA-1 hybridisation localised to a 1kb *Sal* I-*Bam* HI fragment and the adjacent 7kb *Bam* HI fragment (Hasima, 1992), (Fig 5.1). This region was flanked by about 2kb of DNA at its 5'-end and about 12kb at its 3'-end, with respect to the orientation of the gene (Hasima, 1992). The phage DNA from clone 19.1 prepared by the CseI-step gradient method (section 2.3), was digested with *Bam* HI and *Sal* I, the fragments were gel-purified as described in section 2.6.3. The 1kb *Sal* I-*Bam* HI fragment from clone 19.1 was ligated into pBR322 cut with *Sal* I and *Bam* HI to form pBoLA-17. Next, the 7kb *Bam* HI fragment was subcloned in *Bam* HI cut, pBR322 dephosphorylated (section 2.7.1) to form pBoLA-18. Finally, the original arrangement of the fragments was reconstructed in pBoLA-19. The correct orientation of the fragments in pBoLA-19 was then confirmed by *Eco* RI and *Sac* I double digestion.

Fig. 5.1 Restriction map of 19.1 phage clone (from Hasima, 1992)

Clone 19.1 contained a 21kb insert fragment. The 7kb *Bam* HI fragment which hybridised to pBoLA-1 is shaded. The orientation of the gene encoded in 19.1 is as shown in the figure. EMBL3 vector DNA is shown as a thick line while insert DNA is shown by a thin line.



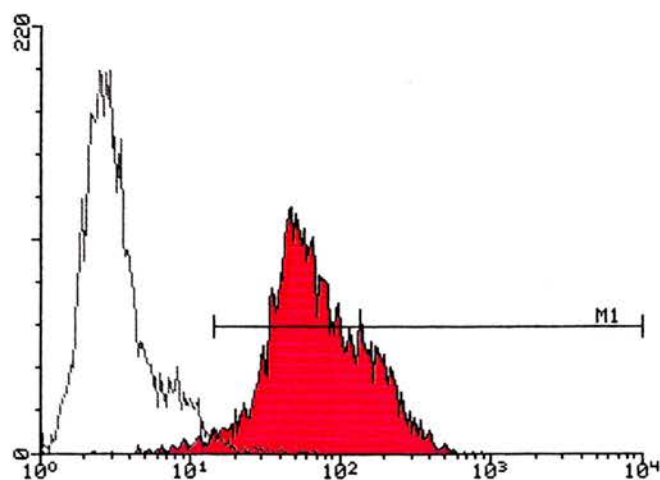
5.3 Transfection of pBoLA-19 in Ltk⁻ cells

In order to show that pBoLA-19 carried the entire class I gene from the phage clone 19.1, it was co-transfected into Ltk⁻ cells with the *tk* gene as described in section 4.2.1. After HAT selection, 90% of the surviving transfectants expressed a bovine class I molecule as detected by FACS analysis with IL-A88 (Fig 5.2). This confirmed the integrity of the gene on pBoLA-19. Transfection of the pBoLA-19 plasmid always gave more than 90% class I expression after transfections, while transfection of the 19.1 phage clone showed only 15-20% cells expressing class I molecules in primary transfection. This difference in the expression levels of primary transfectants might be due to the smaller size of the plasmid DNA compared to the phage DNA which increases the relative concentration of the class I gene transfected. In subsequent experiments both phage and plasmid-derived transfectants from the 19.1 gene gave identical results. From the transfection results of pBoLA-19 it appeared that the 19.1 gene subcloned in pBR322 had all of the flanking 5'- and 3' sequences necessary for transcription of the gene.

5.4 Sequencing of the 19.1 gene.

The sequencing of the 19.1 gene was done from the three plasmids pBoLA-17, pBoLA-18 and pBoLA-19 by the dideoxy chain termination method of Sanger and co-workers (1977), with modified T7 DNA polymerase as described in section 2.8.2. Since the 19.1 gene came from a genomic library, intron sequences were present within the gene. Initial sequencing therefore used primers designed in the conserved regions of the exons from published bovine class I cDNA sequences (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Ellis et al. 1992; Garber et al. 1993) and pBR322-specific primers flanking the cloned fragments.

Fig. 5.2 Flow cytometry analysis of L cells transfected with the 19.1 plasmid clone pBoLA-19. The cells were stained with bovine class I mAb IL-A88 and control Ltk⁻ were stained with IL-A12 which was used as an isotype control for IL-A88.



Extension primers, based on the sequence data obtained, were used to complete the sequences of all of the exons in the class I gene on clone 19.1 including contiguous segments of 1034 base pairs (bp) and 886 bp at the 5' and 3' ends of the gene, respectively (Fig 5.3). The length of the unsequenced introns in the 19.1 genomic clone were subsequently determined by PCR amplification of the introns using flanking exon primers, and subtracting the known distances from the primer sites to the intron boundary. The estimated sizes of the introns are given in the Fig 5.3.

The 19.1 gene has all the characteristic features of an antigen presenting molecule, with conserved cysteines residues to form the disulphide bonds characteristic of the immunoglobulin domain structure, a glycosylation site in the α_1 domain, and the conserved site for phosphorylation in the cytoplasmic domain. The location of the exon/intron boundaries was determined by homology with the published cDNA sequences and the position of splice donor and acceptor dinucleotides AG and GT (Lewin, 1980). The organisation of the pBoLA-19 gene is similar to that determined for the HLA class I genomic sequence of HLA-A2 and HLA-A3, and also to that found for murine class I genes (Strachan et al. 1984).

Fig. 5.3 The nucleotide sequence of the class I gene from the lamda clone 19.1. Intron sequences are given in *lowercase*. The translated amino acid sequences of the exons are given in *three-letter* code above shown in *italics*. The region equivalent to the class I regulatory complex (CRC) sequence in HLA genes is shown in *bold* type, and promoter sequences are underlined and indicated as follows: *CAAT*, CCAAT-box; *TATA*, TATAA box. Predicted polyadenylation sequences are in *bold italics*. Restriction enzyme sites are *overlined* and labelled as follow: *Bam*, Bam HI; *Pvu*, Pvu II. The estimated sizes in bp of unsequenced regions are given in their appropriate positions in *ellipses* (...)

AAAGGATCTTAGTTTCTGAAATGTCAGTTTAAAGCCAACCTTTTAACTCTCTCTTTCACCTTTCATCAAGAGGCTCTTAACTTCACCTTTCGCCATTAAGGTAATTGTCTCTGCATAT	120
CTGAGGTATTGATATATTTCTCCAGCAATCTTGATTCAGCTTGTGCTTCATCCAGTCCAGCAATTTCTCATGAGGTACGCTGCATATTAAGTTAATAATCAGGGTGAACAATATACAGCT	240
TGACATATCTCTTCCAGATTTTGAAAACCAAGTCTGTCTTTTCCATGTCAGTCTTAACTCTTGTCTTGAACCTGCATACAGATTTCTCAGGAGGCAAGGTACAGATGGTCTGTATTCCCAT	360
CTCTTGAAGATTTTTCACAGTTTGTATTGATCCACACAGACAAGGCTTTGGCAATGTCATTAAGAAGAGATGATGTTTTCTCTTTTGTCTTTTTCGATTAATTCAGGGGATTTGGCA	480
CTTTGATCTCTGGCTCTCTGGTTTTCTTAATCCGGCTTGAAGTCTGAAGAATTCATTAATTAAGTACTGTGTAAGCCCTGTGTTTTGAAGATTTTGAAGATTACTTTGTTAGCATGTGAG	600
ATGATGCAATTATTCAGTATGTTGAATATTCTACTAGCAAGAGGAGGATCAGGCAAAAGTCCAGGTCCCTGTGTGGGCAACCGCTCAGGATTCAGAGTCAAGGGCGGTCTGTGGGGC	720
TGGAGCGCTCCAGTGTGGGAGTCCCTCTCTCCCGAGTTTCACCTTCTCTTCACAACACCTGTGTGGCCCTCTGCTAGACACTCATGATGCGGCCCAAGCTCTCACTGGCACTGGG	840
<div style="display: flex; justify-content: space-around; width: 100%;"> <div>CAAT</div> <div>Bam</div> <div>TATA</div> </div> TGTTCAGTTCTAGAAAACCAATCAGCGTCTCCGTGGATCCAGTTATTAAGTCTCCACCGAACCCGCAAGACTCTGTCTTCTTCCAGACCCAGAGGATGCGAGTTATAGGCGCGCAAC	960
<div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 1</div> <div>MetArgValMetArgProArgTh</div> </div>	
rLeuLeuLeuLeuSerArgValLeuValLeuThrngl uThrLeuAlaG <div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 2</div> <div>LySerHisSerLeuArgTyrPheTy</div> </div>	1067
CTTCTCTCTGCTGCTCTGCGGGTCTGTGTTCTGAACGAGACCTTGGCGGtgagtgcgsggtctgagsgaac... 270bp ...tgccccaggtCTCCACTCCCTGAGGTATTTCAT	
rThrAlaValSerArgProGlyLeuGlygl uProArgPheIleAlaValGlyTyrValAlaSpAspThrnglnPheThrArgPheAspSerAspAlaProAsnProArgAspGluProArgVa <div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 3</div> <div>LySerHisThrPheGlnGluMetTyrGlyCysTyrValGlyProAspGlyArgLeuLeuGlnGlyPheMetGlnPheAlaTyrAspGlu</div> </div>	1187
CACCGCGGTGTCCCGGCGCGGCTCTGCGGAGGCCCGCTTCATCGCGGTGCGCTACGTGACGACACGACAGTTTCCGCGGAACTGAACACCGCACCTCGGCTACTACCAACGAGCGCA	
lProTrpMetGluGlnGluGlyProGluTyrTrpAspArgAsnThrArgIleTyrTyrAspThrAlaGlnIlePheArgAlaAsnLeuAsnThrAlaLeuGlyTyrTyrAsnGlnSerGlu <div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 4</div> <div>spProProLysAlaHisVal</div> </div>	1307
GCGGTGATGAGCAGGAGGCGCGCGGAGTATTGGATTCGAAACACGAGAATTCACAAAGGACACCGCACATTTCCGCTACTACCAACGAGCGCA	
uAlaG <div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 3</div> <div>LySerHisThrPheGlnGluMetTyrGlyCysTyrValGlyProAspGlyArgLeuLeuGlnGlyPheMetGlnPheAlaTyrAspGlu</div> </div>	1414
GGGCGgtgag... 200bp ...gcgsggtcaggggtCTCACACCTTCCAGGAGAGTACGGCTGTCTACGTGGGGCGGACCGCGGCTCTCTCTCTGCGGTTCATGCAAGTTCCCTTACGACGCGG	
yArgAspTyrIleAlaLeuAsnGluAspLeuArgSerTrpThrAlaAlaAspThrAlaAlaGlnIleThrTyrArgLysTrpGluAlaAlaGlygl uArgGlnArgAsnTyrLe <div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 4</div> <div>spProProLysAlaHisVal</div> </div>	1534
CAGAGTTTACATCGCCCTGAACGAGGACCTGCGCTCTCTGACCGCGCGGACACCGCGGCTACGATCCAAACCGAAAGTGGAGGCGGAGGTGAGGCGGAGAGACAGAGAACTACTT	
uGluGlyArgCysValGluGlyLeuArgArgTyrLeuGluuAsnGlyLysAspThrLeuLeuArgAlaA <div style="display: flex; justify-content: space-around; width: 100%;"> <div>exon 4</div> <div>spProProLysAlaHisVal</div> </div>	1640
GGAGGGCGGGTGCGTGAGGCGGGCTCCGACGATATCTGAGAGACGGAAAGGACACGCTGTCTGCGCGCAGgtacgaggg... 900bp ...cctcctcagAACCTCCAAAGGACACTGTG	

The sequencing primers used, and their locations on the 19.1gene, are given in the Table 5.1:

Primer	Location	Sequence
4477	exon 1 reverse	5' GTTCCCTCCAGACCCCGC 3'
1573	Repetitive forward	5' CAGCATTTCTCATCATGAGGT 3'
1572	promoter reverse	5' ACCGCCCTTGACCT 3'
770	exon 2 forward	5' GCTCCCACTSMTGAGGTATT
771	exon 2 reverse	5' CTCGCTCTGGTTRTAGTAGCC 3'
1595	exon 3 forward	5' GGCTGCKACGTGGGGCC 3'
1596	exon 3 reverse	5' TCCAGGTATCTGCGGAGC 3'
1597	exon 4 forward	5' CCTCCAAAGGCACATGT 3'
1598	exon 4 reverse	5' GGCTCCTGAAGCCCC 3'
1599	exon 5 reverse	5' CCTCAGMCTCCTTCCTC 3'
4482	intron 5 reverse	5' TACCTAGGAATGGAAACC 3'
1600	exon 7 reverse	5' AGACACATCAGAGCCC 3'
773	exon 7 forward	5' GGCTCTGATGTGTCTCTCACG 3'
772	exon 8- 3'ut reverse	5' GATGMAGCATCACTCAGTCCC
1601	3'-ut forward	5' GGGGGACTGAGTGA 3'
4476	3'-ut- poly A forw	5' TCCCCACCCTGACCTGTGTTC 3'

5.4.1 General features of the sequence

The leader sequence, exon 1: Two in-frame initiation codons located at nucleotides 938 and 947 of the pBoLA-19 sequence result in two possible leader peptides of 21 and 24 amino acids. This is similar to the organisation observed for the HLA-A2

gene (Koller and Orr, 1985; Sodoyer et al. 1984). The mature pBoLA-19 antigen is encoded by exon 2- exon 8. Each of the three extracellular domains of mature pBoLA-19 protein is encoded by a separate exon ie exon 2- exon 4. Exon 5 encodes the hydrophobic transmembrane segment which contains of about 35 amino acids. Exons 6-8 form the cytoplasmic tail of the molecule containing mainly hydrophilic amino acids.

5.4.2 *The promoter region*

Transcription of class I genes is controlled by regulatory elements residing in the 5' flanking region (David-Watine et al. 1990). In addition to the TATA and CAAT box sequences necessary for RNA polymerase II positioning, the MHC class I promoter contains an upstream enhancer region (EnhancerA) that partially overlaps the Interferon Response Sequence (IRS: Kimura et al. 1986). The EnhancerA/IRS is necessary for class I transcription modulation through cytokine stimulation (Harms and Splitter, 1992).

The 5' flanking sequence of the 19.1 gene was found to be homologous with MHC class I promoters, containing an identifiable class I regulatory complex (CRC and promoters elements). The "CAT" box (CCAAT) is found 74 base pairs upstream to 5' of the initiation codon. Approximately 20 base pairs (bp) down-stream from the CAT box, the 19.1 sequence contains a canonical "TATA" box (TATAA). These elements are believed to be crucial for the accuracy and efficiency of transcription by RNA polymerase II (Breathnach and Chambon, 1981). The upstream region to CAAT and TATA boxes contained a 12bp palindromic sequence TGGGGATTCCCC which was identified as a possible binding site for the nuclear transactivating factor. This consensus sequence is also found conserved in the H2-K^b and HLA -B7 (Israel

et al. 1987; Baldwin and Sharp, 1988). Another regulatory motif found was the interferon responsive element (IRE) AGTTTCACTTTC. The IRE in class I genes is necessary for stimulation by interferon (IFN) and seems to be conserved in various class I sequences.

There are only two other bovine class I promoter sequences available in the database which were used for comparative analysis of the 19.1 promoter. The degree of the identity between the 241bp CRC of pBoLA-19 and cattle promoter sequences BTMHCI (J.S. Harms, unpublished data; accession L19193) and BoLAenh-9 (Harms and Splitter, 1994) is 92% and 86% respectively. In man, the CRC sequences are highly conserved between alleles of a single locus (96%- 99% identity), but are less conserved between loci (78-89% identity; Cereb and Yang, 1994). These comparative results in humans suggests that pBoLA-19 and BTMHCI promoters may arise from different loci. This is further supported by the observation that the exon 1 sequence of BTMHCI is identical to that of the cDNA BL3-6, while pBoLA-19 appears to be allelic with BL3-7. The BL3-6 and BL3-7 cDNAs were suggested to be from different loci on the basis of the length of the transmembrane region (Ennis et al. 1988).

5.4.3 5' upstream region

The pBoLA-19 sequences upstream of exon 1 can be split into two distinct areas based on the similarity to known sequences. The region from position 1-630 showed a high degree of homology (84-86%) with sequences within the bovine lysozyme gene, gamma globin and α_2 casein, in searches of the EMBL nucleotide sequence database. On further analysis it was found that this region is similar to the *Art2* repetitive element in cattle lysozyme genes (Irwin et al. 1993), suggesting that this

region may be part of the repetitive element. The variation in length of the three cow lysozyme genes appears to be due to the presence or absence of these repetitive DNA within the introns of the genes. The *Art2* repetitive element has been identified in the goat in addition to the cow (Spence et al. 1985; Duncan, 1987; Li et al. 1991).

5.4.4 *Analysis of the coding region*

The analysis of the nucleotide sequence and amino acid sequences of the 19.1 gene are presented as a comparative analysis in chapter 8.

5.5 Discussion

The 19.1 genomic clone was analysed by restriction mapping, and a 7.0kb *Bam* HI fragment was shown to contain a class I gene by Southern blotting. The expressed product from this gene was characterised by serology, IEF and by CTL assays and encoded a component of the BoLA-A11 haplotype. Genetic information about the cattle MHC class I has been limited to studies of cattle class I cDNA sequences and little is known regarding the size of the introns and the flanking sequences which control expression. All other BoLA class I sequences available are from the cDNA clones which do not provide any information regarding the introns and flanking regulatory sequences. These regions are useful in comparative analysis of class I genes since they are less well-conserved between alleles of different loci than the exon sequence. In addition, the size of the introns are also much less conserved between species. The length of the first intron from the 19.1 gene was approximately 200bp, which compares with 100bp in human and 350bp in swine. The pBoLA-19 promoter was found to be allelic to BoLAenh-9 (Harms and Splitter, 1994), and from a different locus to BTMHCI based on their degree of similarity.

This observation of locus-specific and allelic variation seen in CRC regions may provide a structural basis for modulation of transcription as seen for HLA class I genes (Cereb and Yang, 1994). The sequence immediately upstream of the class I promoter in pBoLA-19 (Sawhney et al. 1995) appears to contain a repetitive DNA element which clearly defines the start of the class I gene. This region could be used to design primers for locus-specific amplification of the adjacent class I gene. As more bovine class I flanking sequences become available, this approach could be very useful in genetic analysis of different class I loci and in rapidly assigning sequences to different alleles or genes.

Chapter 6

CONSTRUCTION OF CLASS I GENE EXPRESSION VECTOR

6.1 Introduction

The plasmid construct pBoLA-19 which contained the entire class I gene from the phage clone 19.1 showed over 90% class I expression on transfection. From the experimental data on the transfection and sequence of the 19.1 gene, it appeared to contain all of the upstream regulatory and promoter sequences necessary for class I gene expression (Sawhney et al. 1995). The pBoLA-19 plasmid was therefore used as the basis for the construction of a class I gene replacement vector.

The lack of class I gene expression on transfection of the three transfected phage clones 1.3, 15.2 and 17.3x has been discussed in the section 4.4. In order to rule out the possibility that these clones did not express because they were truncated at the 5' or 3'-ends of the gene, the subcloning of these clones was undertaken. The aim of this reconstruction was to replace the coding region of the pBoLA-19 gene with the corresponding gene fragment from the three non-expressible phage clones to provide each gene with a functional class I promoter and 3'-ends to test their expressibility.

6.2 Cloning strategy

In order to reconstruct the phage clones, a 3.2kb fragment which contained the coding region from exon 2 to the 3'-UT region of the class I genes in these clones was amplified using primers based on sequences conserved in exon 2 and in the 3'-untranslated region of the published bovine class I cDNAs (Fig 6.1). To subclone these fragments so they could be expressed, a vector acceptor fragment with a functional class I promoter and 3'-end was first made. This vector fragment was

Fig.6.1 Multiple nucleotide sequence alignments illustrating the selection of primers for amplification of MHC class I gene fragments. In the alignments residues identical to the pBoLA-19 sequence are represented by *dashes* (-), regions of sequence which were not determined are shown by *dots* (.), and gaps inserted to maximise similarity in the alignment are shown by *carets* (^). The sequence of primer 2407 and the reverse complement of primers 2406 and M0700 are given beneath the alignments. Sequences in *italics* in primers 2407 and M0700 are restriction enzyme sites included to facilitate subcloning of the PCR products, and the following ambiguity codes represent degenerate positions in the primer sequences: S, G or C; M, A or C; Y, C or T; and W, A or T.

	Intron 1	Exon 2
BL3-7	-----
BSF	-----
BSN	-----
BL3-6	-----
BSA	-----
W10	ga-----
pBoLA-1	ga-----
BSC	-----
KN104	-----
pBoLA-19	gtctcaccct cttgccccag	GCTCCCACTC CCTGAGG

GAGGTCCGGCCG cttgccccag GCTCCCACTC SMTGA
Primer 2407

W10	-----TGCTG	CCCTTCCCCA	C---GT---	-----	-----	---T----
BL3-6	-----TGCTG	CCCTTCCCCA	C---GT---	----G----	-----	---T----
pBoLA-1	-----TGCTG	CCCCTCCCCA	CCCTGAC-..
KN104	-----TGCTG	CCCCTCCCCA	C---...---	-----	-----T----	---T----
BL3-7	-----	G-----	-----	-----	-----
pBoLA-19	CCCAC.....C	ATGCC..CCC	TCCCCACCCT	GACCTGTGTT	CTCCTCCC

CCCACTGCTG CCCYTCCCCA CGGCCGCCCC
Primer M0700 CCC TCCCWACCCT GACCGGTGTT CTCYTCC
Primer 2406

generated from the pBoLA-19 construct. To facilitate this cloning, both vector and insert fragments were amplified using primers designed with terminal restriction sites to allow directional cloning of the class I gene fragments.

The construction of the expression system is outlined in Fig. 6.2, and is summarised below.

6.2.1 *Generation of the acceptor vector from pBoLA-19*

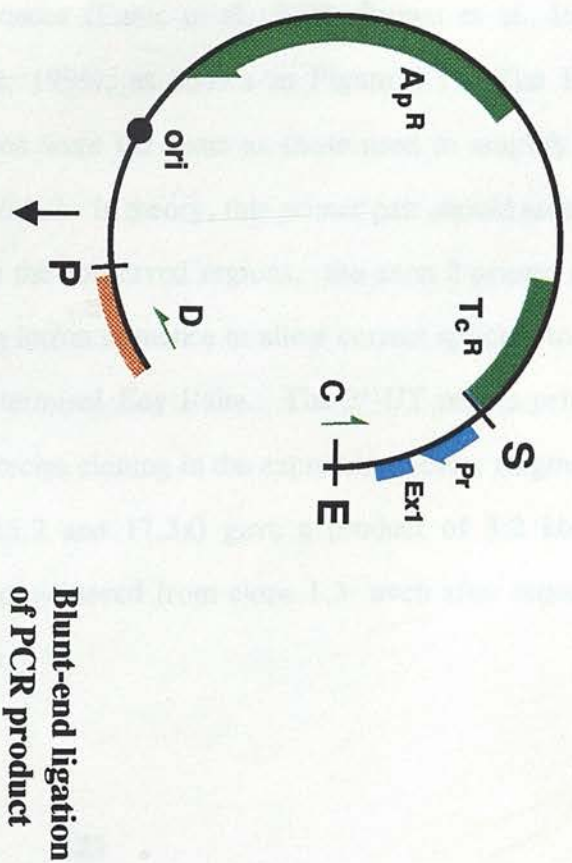
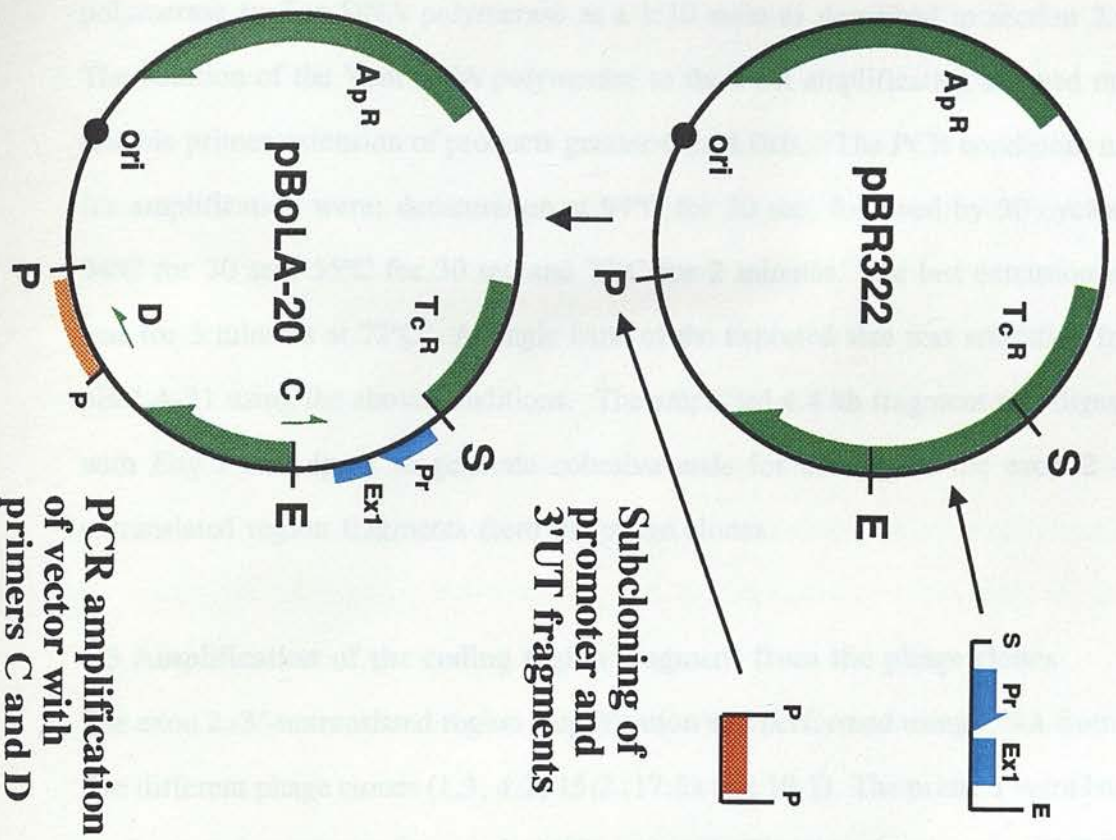
A 1.2 kb *Sal* I- *Eag* I fragment from pBoLA-19 containing 5'-flanking sequences, the class I promoter, exon 1 and 150 bases of the first intron was gel-purified and subcloned into *Sal* I- *Eag* I cut pBR322 to make pBoLA-20. Next, a 450bp *Pvu* II fragment from pBoLA-19 containing most of the 3'-untranslated region and the putative poly-adenylation sequences was subcloned in pBoLA-20 by blunt-end ligation as described in section 2.5.2 to make pBoLA-21 (Fig 6.2). The correct orientation of the *Pvu* II fragment was checked by *Pvu* II and *BstE* II double digestion. pBoLA-21 carried the 5' and 3'-ends of the 19.1 gene, and was used as a template for PCR amplification of the expression vector receptor fragment with appropriate restriction sites at the ends to allow directional cloning of the exon 2- 3'-UT fragment.

6.2.2 *PCR amplification of class I expression vector*

The receptor fragment was generated by PCR from pBoLA-21 using a reverse primer (2405) in intron 1 which included the *Eag* I site, and a forward primer in a conserved region of the 3'-UT region. The 3'-end oligo (2408) had a single base (T/G) mismatch from the original pBoLA-21 sequence to create an *Age* I restriction site at the end of the vector receptor fragment. PCR conditions were modified for the amplification of this large (4.4kb) product by the addition of Deep vent DNA

Fig. 6.2 The expression vector pBoLA-21 was constructed as follows: **(A)** a 1.2kb *Sal* I- *Eag* I fragment containing the class I promoter and exon 1 was subcloned from pBoLA-19 into pBR322. Next, a 450bp *Pvu* II fragment from pBoLA-19 containing most of the 3'-untranslated region and polyadenylation sequences was added to make pBoLA-20. **(B)** Inverse PCR using primers C and D (marked by arrows) was then used to amplify a 4.4kb receptor fragment from pBoLA-20. Primer C was based on intron 1 sequences flanking the *Eag* I site, and primer D was the reverse complement of primer B. Finally, the PCR fragment amplified from pBoLA-20 was self-ligated to make pBoLA-21.

Restriction enzyme sites, and important features of the sequences are shown as follows: S, *Sal* I; E, *Eag* I; P, *Pvu* II; Pr, class I promoter region; Ex1, pBoLA-19 exon 1; Ap^R, Ampicillin resistance gene; Tc^R, Tetracycline resistance gene; ori, pBR322 origin of replication.



polymerase to *Taq* DNA polymerase at a 1:10 ratio as described in section 2.18. The addition of the Vent DNA polymerase to the PCR amplification allowed more reliable primer extension of products greater than 4.0kb. The PCR conditions used for amplification were: denaturation at 94°C for 30 sec, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 minutes. The last extension step was for 5 minutes at 72°C. A single band of the expected size was amplified from pBoLA-21 using the above conditions. The amplified 4.4 kb fragment was digested with *Eag* I and *Age* I to generate cohesive ends for cloning of the exon 2 -3'-untranslated region fragments from the phage clones.

6.3 Amplification of the coding region fragment from the phage clones

The exon 2 -3'-untranslated region amplification was performed using DNA from all five different phage clones (1.3, 4.2, 15.2, 17.3x and 19.1). The primers were based on conserved sequences in exon 2 (2407) and in the 3'-untranslated region (2406) of the published bovine class I sequences (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Garber et al. 1993), as shown in Figure 6.1. The PCR conditions used in the amplifications were the same as those used to amplify the vector acceptor fragment in section 6.2.2. In theory, this primer pair should amplify all class I sequences. In addition to the conserved regions, the exon 2 primer also included 10 bases of the 5'-flanking intron sequence to allow correct splicing to the vector-encoded first exon, and a terminal *Eag* I site. The 3'-UT region primer included a terminal *Age* I site for precise cloning in the expression vector fragment. Four phage clones (19.1, 4.2, 15.2 and 17.3x) gave a product of 3.2 kb on amplification. Amplification was not achieved from clone 1.3 even after repeated PCR under a variety of conditions.

Since both the amplified vector and insert DNAs had terminal *Eag* I and *Age* I restriction sites, the strategy was to digest the PCR-amplified vector and insert DNA fragments with *Eag* I and *Age* I, and then ligate the fragments to yield the reconstructed class I gene(s). The vector and insert PCR products were purified with the Wizard DNA clean-up system to remove *Taq* DNA polymerase and excess nucleotide to facilitate the subsequent digestion and ligation reactions. However, this approach of ligating the restriction-digested PCR fragments did not work. It was speculated that the *Age* I site introduced at one end of the insert and vector fragments might have been refractory to digestion and so rendered this cloning inefficient.

6.4 Change in strategy

A new strategy was worked out to use only the *Eag* I sites to make the cloning exercise effective. In this approach, the 4.4 kb vector fragment amplified from pBoLA-21 was self-ligated. The recircularised DNA molecule was then transformed in *E. coli* DH5 α , and transformant colonies were tested for the recircularised plasmid carrying the *Eag* I and *Age* I sites (pBoLA-21). Plasmid pBoLA-21 gave 1.2kb fragments on digestion with both *Sal* I -*Eag* I and *Sal* I -*Age* I, which showed that the ends of the PCR amplified vector fragment were correctly ligated. This also further demonstrated that the *Eag* I and *Age* I restriction sites were separated by only few bases of intervening primer sequences. This plasmid, which carried the 5'- and 3'-ends of the 19.1 gene flanking a unique *Eag* I site, was used as the acceptor for class I gene reconstruction.

6.4.1 Subcloning of exon 2-3'-untranslated fragment in TA cloning vector

To utilise the *Eag* I cloning site in pBoLA-21, the exon 2 - 3'-UT insert fragments from the phage clones (4.2, 15.2, 17.3x and 19.1) were TA subcloned as described in section (2.7.3). The presence of *Eag* I sites in the polylinker of TA cloning vector pCRII facilitated the *Eag* I subcloning of the inserts into pBoLA-21. The orientation of the class I gene fragment in pCRII was not critical for subcloning in pBoLA-21 since the pCRII polylinker has two *Eag* I sites flanking the cloning site. The TA clones were screened by colony PCR as described in section 2.18.1 using exon 2 -specific primers (770 and 772). Those clones which gave product of 270 bp were used to prepare plasmid minipreps for further analysis. The plasmid DNA prepared from these TA clones was digested with *Eag* I to determine the size of the insert fragment. Four TA clones carrying the 3.2kb class I gene fragment were obtained for each of the phage clones. Plasmid DNA from the four TA clones representing each individual phage clone were combined in equimolar amounts, digested with *Eag* I, and the 3.2kb insert fragment was purified from agarose gels. The 3.2kb fragment representing each phage clone was then subcloned into *Eag* I-digested pBoLA-21.

6.4.2 Reconstruction process

In order to reconstruct functional class I genes from the phage clones, pBoLA-21 DNA was *Eag* I digested and dephosphorylated with Calf intestinal phosphatase as described in section 2.7.1, before ligation with the *Eag* I digested 3.2kb insert fragments. The correct orientation of insert fragment in the reconstructed clones was determined by *Sal* I- *Pvu* II double digests. Clones with the correct orientation gave a 3.2 kb *Sal* I- *Pvu* II fragment, whereas a 1.2 kb fragment was seen in clones with the wrong orientation.

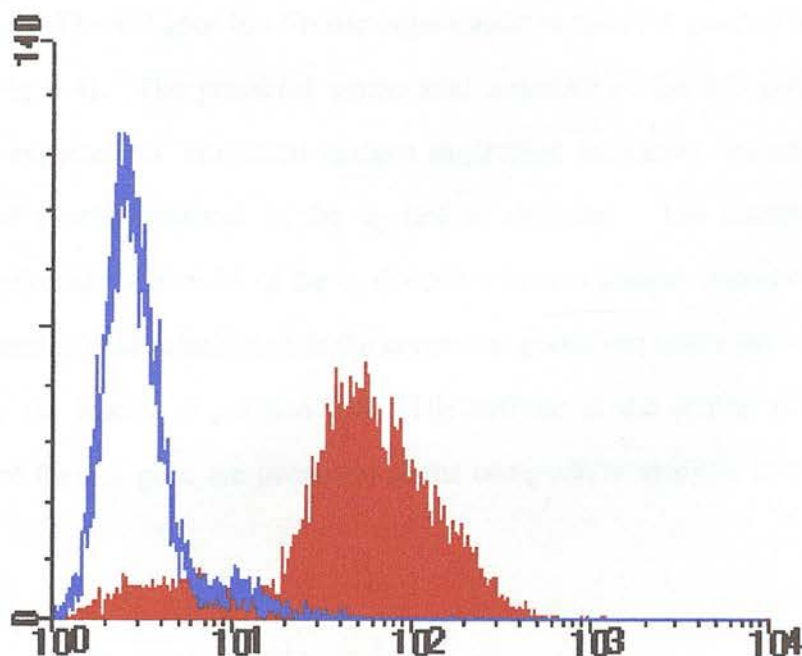
6.5 Transfection of reconstructed phage clones

Representatives of the four reconstructed phage clones (19.1, 4.2, 15.2 and 17.3x) pBoLA-25, pBoLA-4, pBoLA-27 and pBoLA-28 were transfected as described in section 4.2.1. The reconstructed version of clone 19.1 in the pBoLA-25 plasmid expression constructs showed more than 90% of HAT resistant transfectants expressing class I molecules (Fig 6.3). The second positively-expressing phage clone, 4.2, when transfected as a reconstructed version in pBoLA-4 also expressed as expected. The two clones 15.2 and 17.3x which did not express in earlier experiments were again found to be negative for class I expression even after reconstruction in the expression vector. These two clones are most likely to be pseudogenes.

6.6 Flipping of *Sal* I-*Pvu* II fragment from pBoLA-21 into pBR322

The reconstructed phage clones (4.2, 15.2, 17.3x and 19.1) in the pBoLA-21 expression construct had clockwise orientation of the class I gene with respect to the pBR322 sequences, whereas the orientation of the class I genes in pBoLA-19 construct was anti-clockwise. In order to investigate that whether the orientation of the class I genes had any influence on the expression, a 1.6kb *Sal* I-*Pvu* II fragment containing the class I promoter, exon 1 and 3'-untranslated sequences from pBoLA-21 was subcloned into *Sal* I- *Eco* RV digested pBR322 to reverse its orientation and make pBoLA-22. This was done to mimic the orientation of the 19.1 gene in pBoLA-19 to determine whether flanking plasmid sequences had any effect on the expression of the gene after transfection. The 19.1 reconstructed class I gene in pBoLA-26 showed similar levels of the class I gene expression on transfection to those observed in pBoLA-19 and pBoLA-25 in earlier experiments.

Fig. 6.3 Fluorescence-activated cell scan (FACS) analysis of bovine class I molecules expressed on the surface of transfected cells. Cells were labelled with the anti-bovine class I monoclonal antibody IL-A88, followed by a fluorescently-labelled anti-mouse IgG secondary antibody. After washing, the degree of cell fluorescence is plotted against cell number for both the negative control (untransfected L cells, blue outline curve) and for the reconstructed pBoLA-19 class I gene in pBoLA-21 (red curve).



6.7 Sequencing of the 4.2 gene

The 4.2 gene was amplified as an exon 2- 3'-UT fragment from the phage clone, and was subcloned in pBoLA-21 to make pBoLA-4. DNA sequencing was done from this reconstructed plasmid version of the 4.2 clone which showed expression in L cells. The sequencing primers designed for the sequencing of the 19.1 gene was used for initial sequencing of the 4.2 gene. In addition, new primers were designed to complete regions of exon sequences which were not read by the earlier primers (Table 6.1). The 4.2 gene has similar organisation to the 19.1 gene of its introns and exons (Fig 6.4). The predicted amino acid sequence of the 4.2 gene has all the features expected of functional antigen presenting molecules including the four conserved cystine residues in the α_2 and α_3 domains. The conserved site for glycosylation at position 86 of the α_1 domain was also present. However, there was a single amino acid substitution in the conserved phosphorylation site, with arginine replacing the leucine at position 334. The analysis of the coding and non-coding regions of the 4.2 gene are presented in the comparative analysis in chapter 8.

Table 6.1 The sequencing primers used and their location on the 4.2 gene.

Primer	Location	Sequence
770	exon 2 forward	5' GCTCCCACTSMTGAGGTATT 3'
771	exon 2 reverse	5' CTCGCTCTGGTTRTAGTAGCC 3'
N0700	exon 2 forward	5' GAGGGGCYSGAGTATTGG 3'
N0701	exon 2 reverse	5' CCAATACTCSRGCCCTC 3'
1595	exon 3 forward	5' GGCTGCKACGTGGGGCC 3'
1596	exon 3 reverse	5' TCCAGGTATCTGCGGAGC 3'
N0699	exon 3 reverse	5' CCGCGGTCCAGGAGYGCA 3'
N0698	exon 3 forward	5' TGCRCCTCCTGGACCGCGG 3'
1597	exon 4 forward	5' CCTCCAAAGGCACATGT 3'
5804	intron 4 forward	5' GAGCCCTTCAGGACTCAG 3'
4659	intron 5 reverse	5' GACCAGGCCTTCAGGGGA 3'
4660	exon 4 forward	5' ACCTGAGCGCTTCTTCMT 3'
1600	exon 7 reverse	5' AGACACATCAGAGCCC 3'
773	exon 7 forward	5' GGCTCTGATGTGTCTCTCACG 3'
772	exon 8- 3'-ut rev	5' GATGMAGCATCACTCAGTCCC 3'
1601	3'-ut forward	5' GGGGGACTGAGTGA 3'

Fig. 6.4 The nucleotide sequence of class the I gene from the lamda clone 4.2. Intron sequences are given in *lower case*. The translated amino acid sequences of the exons are given in *three-letter code* above the DNA sequence. The estimated sizes in bp of unsequenced regions are given at their appropriate positions in *ellipses* (..) conserved phosphorylation site, with arginine replacing the leucine at position 334. The analysis of the coding and non-coding regions of the 4.2 gene are presented in the comparative analysis in chapter 8.

exon 2	
cgagaggtccgcccgttgccccagggctcccactgcgaggtatttcttcaccccgctggtcccgccccaacctggggagcccccgtacctggaagtcggctacgt	120
LaspAsnThrGlnPheValArgPheAspSerAspAlaArgAsnProArgMetGluProArgAlaArgTrpValGluGlnGluGlyProGluTyrTrpAspGlnAsnThr	
GGACAACACGACAGTTGTCGGTTGCAGAGGACGCCCGGAATCCAGAGATGAGCCGCGCGGTTGGTGCAGAGAGGGGCCGAGTATTGGGATCAGAACACG	240
ArgAsnSerLysGlyAlaAlaGlnThrPheArgValAspLeuAsnThrLeuArgGlyTyrTyrAsnGlnSerGluAlaG	
CGAAACTCCAAAGGCGCGCCGACAGACTTTCGAGTGGACTTGAACACCCCTGCGGCTACTACACACAGAGCGCGtgtagcgacgcgccggtccaggtcacga	360
exon 3	
cccccatccccagggaccgcgsgtgcgccgagtcacgsgtcgaagtgt...270bp...cagatttcagtttggtttaatcacgcgcggtggtcgsggcggtcaggggt	471
exhiSThrTl eGlnAlaMetTyrGlyCysAspValGlyProAspGlyArgPheLeuArgGlyTyrArgGlnAspAlaTyrAspGlyArgAspTyrTl eAlaLeuAsnGln	
CTCAACTATCCAGGCGGATGTACGGCTGCGAGTGGGCGGACCGGCGCTTTCCTCCGCGGTTACAGGCAAGAACGCTTACGACGGCAGAGATTACATCGCCCTGAACGA	591
uAspLeuArgSerTrpThrAlaAlaAspThrAlaAlaGlnTl eThrTysArgLysTrpGluGluAlaGlyAlaAlaGluGlyGluuArgAsnTyrLeuGluGlyThrCys	
GGACTGCGCTCTGGACCGCGCGGACACGCGCGCTCAGATCACCAAGCCGCAAGTGGAGAGGCAAGGTGCTGCGGAGGCGCAGAGAACTACTGGAAGGCGACGTGC	711
ValGluThrLeuArgTyrLeuGluuAsnGlyLysAspAlaLeuLeuArgAlaA	
GTGAGTGGCTCCGAGATACCTGGAGAACCGGAAAGGACCGCTGCTGCGCGCAGtaacgagg...200bp...gatggtcacataatgctgcttcagtgaccatgga	822
exon 4	
spProProlYsAlaHi sValThrHi sHi sProll eSerAspArgGluValThrLeuArgCysThrPalaleuGlyPhe	
accacaactgaattttctgatcttcctcagAACCTTCCAAAGGCACATGTGAACCATCAACCCATCTTGACCGTGAAGGTCAACCTGAAGGTGCGCCCTGGGCTTC	942
TyrProGluGluTl eSerLeuThrTrpGlnArgGluGlyGluAspGlnThrGlnAspMetGluLeuValGluThrArgProSerGlyAspGlySerPheGlnLysTrp	
TACCTGAGGAGATCTCACTGAACCTTGGCAGCGCGAGGGGAGGACCAACCCAGGACATGGAGCTTGTGGAGAACGACCTTCAAGGGGACGGAAGCTTCCAGAAAGTG	1162
AlaAlaLeuValValProSerGlyGluGluGlnArgTyrThrCysArgValGlnHi sLysGlyLeuGlnGluProLeuThrLeuTl eTrpG	
GCAGCCCTGTGTGCTTCTTGGAGAGGAGCAGAGATACAGTGCCTGTGTGCAGCACAAAGGGCTTCAGAGAGCCCTCAACCTGATTATGGgttaagggggaatcg	1282

gaatgagcttcctcctcagaaagcagagccctcaggaactcagtcagcaagtcaggaactcaagttgagtcagggagcttcccttccaccagAACCTCCTC	exon 5 InProProG	1402
InProSerValProIleMetGlyIleValValGlyLeuValLeuLeuValAlaValAlaGlyAlaValIleTrpArgLysCysSerg		1522
AGCCCTCCGTTCCCATCATGCGCATCGTTGTGTGGCTGTGTTCTCTCTGTGTGGCTGTGTGTGAGCTGTGATCTGAGGAAGAAGTGTCAGgtagggaaagga		1522
gggagggcctgaatttct..225bp..ctcagccttctccctggcctcaccgtgatatttctctccacagTGAATAAGGACAGACCTACACTCAGGCTGCAGta	exon 6 LysIleLysGlyGlnThrTyrThrGlnAlaIle	1633
agtaLtgagggagtgatccctcgagacccttgttgtggtgcagacagggagg..40bp..gtagtctaccacatccctggtctgttctcccccaaggCAGTGCAGTGC	exon 7 SerAspSerAsp	1745
GlnGlySerAspValSerArgThrValProLysV		1865
CAGGCTCTGATGTGTCTCGCACGGTTCTTAAAGtgagaccacaggaaggactacattbgcaaggggattgggacagaggggacacatbtgggtgctggggatcttgag		1865
tgggacatgtgagcatgtggggggctgtgaagaatgtcagcccttacatgactgacctaaactggttcctgattctttctctccacagTGTGACACTGCTTGTGTGGG	exon 8 allEnd	1985
ACTGATGATTCGCATCCCGCATAGTACATCAAGATCTCCGGACCCCTCTTTCTGCACTGCATCGAACGTCGTGCTTTCTAGTAGAGGAAGCACACATGA		2105
GGAGCTGGGGAAGCTGCCCCCTTCCCAACATGCCCCCTCCCCCACTGACCGGTTCCTCCCAAGCCGAATTCCAGCACACTGCGCGCGGCTCTCCCC		2222

6.8 Discussion

By reconstructing the phage clones it was possible to answer some of the questions which were raised by the phage transfection results. The failure to amplify the exon 2 -3'-untranslated fragment of clone 1.3 with primers 2406 and 2407, appeared to be due to primer 2407 in the 3'-untranslated region, since the exon 2 forward primer (2406) marked in conjunction with a different 3'-end reverse primer (772), which annealed 100 bases upstream of primer 2407. This suggests that clone 1.3 might be truncated at the 3'-end of the gene, or might have different sequence at the 2407 primer site which could have resulted in failure of this primer to anneal. This clone could have been reconstructed using the 2406 and 772 primers which would amplify 100bp shorter product, but due to lack of time it this could not be done.

The PCR amplified vector and insert fragments could not be ligated together after *Eag* I- *Age* I double digestion. The reasons for this are not clear, but it may be because the restriction sites incorporated in primers were close to the ends of the primers, at a distance of five or six nucleotides from the end. It is unknown whether this is sufficient to allow efficient digestion with *Eag* I or *Age* I. This might be one reason that restriction enzymes did not cut efficiently to make cohesive ends for ligation.

The two plasmid expression constructs, pBoLA-25 and pBoLA-26 which had different orientations of the class I promoter in the plasmid, were found to behave in a similar fashion in transfection assays of clone 19.1, suggesting that flanking DNA sequence had little influence on the expression of the gene. The other three clones were not reconstructed in pBoLA-22, since no difference was observed in the

level of expression of the two versions of the 19.1 gene.

The two non-expressing clones (15.2 and 17.3x) that could be amplified with primers 2406 and 2407 did not express after reconstruction, suggesting that these clones are likely to carry pseudogenes. It is not surprising that two phage clones from the group of five appeared to be pseudogenes. The number of pseudogenes in human class I region have been observed to be quite significant (Klein, 1986). It has been proposed that these genes may provide a source of donor sequences for generation of variation in classical class I genes (Lawlor et al.1990).

The expression system devised here for analysis of genomic clones can also be used for the cloning and expression of cDNAs. Furthermore, the class I promoter in the pBoLA-21 vector is flanked by unique *Sal* I and *Bam* HI restriction sites which would allow the replacement of the promoter if increased or tissue-specific expression were desired.

Chapter 7

CLONING AND EXPRESSION OF cDNA CLONES IN pBoLA-21

7.1 Introduction

The primers used for amplification of class I genomic fragments from the phage clones were based in transcribed regions of the gene and so could be used for cDNA amplification as well. After recloning and successfully expressing class I gene fragments from the coding regions of two positively-expressing genomic clones (19.1 and 4.2) in pBoLA-21, the expression of cDNA clones was tested in pBoLA-21. In order to characterise the expression potential of the pBoLA-21 vector for cDNA clones, a plasmid-encoded bovine class I cDNA available in the laboratory (pBoLA-1; Brown et al. 1989) was subcloned into pBoLA-21. The pBoLA-1 cDNA clone is truncated at both ends of the gene, which would make it difficult to express in standard systems without considerable manipulation. In general, full length cDNA clones are required for expression. However, since the approach used here involved only the subcloning of sequences from exon 2 to 3'-ut into the expression cassette, it should make the pBoLA-1 cDNA expressible.

7.2 PCR amplification of pBoLA-1 cDNA

The exon 2 -3'-UT fragments from the 19.1 and 4.2 genomic clones were amplified using the exon 2 forward primer 2406, and the 3'-UT reverse primer 2407. The amplified fragments were then subcloned into a TA vector to generate *Eag* I ends for cloning into the unique *Eag* I site of pBoLA-21.

To subclone and express the pBoLA-1 cDNA in pBoLA-21, an exon 2- 3'-UT fragment for pBoLA-1 class I cDNA was amplified from its plasmid-encoded recombinant clone. For PCR amplification, the exon 2 forward primer (2406)

previously used to amplify the 3.2kb class I gene from genomic clones was used. However, a different reverse primer (M0700) was required because pBoLA-1 is truncated at the 3' end and does not include the primer 2407 site. Primer M0700 was based at the end of the pBoLA-1 cDNA sequence to include the maximum 3'-untranslated sequence. This primer had a 15bp sequence conserved in four of the published bovine cDNA sequences BL3-6, pBoLA-1, A10 and KN104 (Ennis et al. 1988 and Bensaid et al. 1991) and a terminal *Eag* I site (Fig 6.1). PCR conditions were: denaturation for 94°C for 90 sec, followed by 10 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 minutes. A further 20 cycles were then done with an annealing temperature of 55°C. The lower annealing temperature was used during the initial cycles to allow the exon 2 forward primer (2406) to anneal sufficiently. This primer had only the first 12 bases of exon 2 to anneal with the pBoLA-1 cDNA as the rest of the primer contained flanking intron sequences and an *Eag* I site for cloning into pBoLA-21. In order to increase the yield of the specific PCR amplified product the annealing temperature was raised from 50°C to 55°C for the last 20 cycles using these conditions. A major band of 1.2kb was seen on agarose gels along with some smaller size bands. The 1.2kb amplified product contained the exon 2-3'-UT insert fragment from pBoLA-1 cDNA.

7.3 Reconstruction of the pBoLA-1 amplified cDNA

The 1.2kb amplified pBoLA-1 cDNA contained terminal *Eag* I sites which were based in the 5' and 3'-end primers and was subcloned into *Eag* I site of the expression vector pBoLA-21, to make pBoLA-30. The correct orientation of the insert in pBoLA-30 was determined by *Sal* I-*Pvu* II double digestion. The reconstructed pBoLA-1 cDNA in pBoLA-30 was transfected into L cells as described in section 4.2.1 to test its expression.

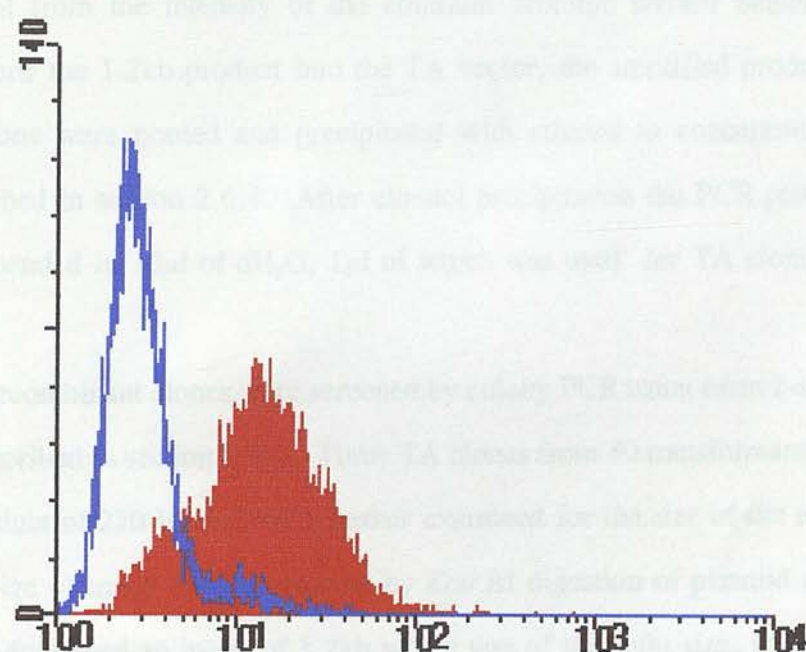
7.4 Transfection of the reconstructed pBoLA-1 cDNA clone (pBoLA-30)

The primary transfectants obtained from pBoLA-30 showed a very low percentage of cells positive for bovine class I molecules when analyzed with IL-A88. After two MACS sorts, however, a separate peak of class I- positive transfectants was evident (Fig 7.1). This results suggests that the expression vector pBoLA-21 can express coding sequences from cDNA clones in addition to genomic class I clones. In order to further characterise functional class I genes from the A10 and A11 haplotypes, the primers 2407 and M0700 were used to amplify class I genes from crude cDNA made from total cellular RNA for cloning (into pBoLA-21) and expression. This experiment is described below.

7.5 RT-PCR amplification of bovine class I cDNA with A10-specificity

The data from the 19.1 and 4.2 transfected cell lines demonstrated that both these clones had BoLA-A11 specificity. These phage clones had been isolated from a genomic library prepared from an animal (10769) with A10 and A11 serotypes, but a functional class I genomic clone corresponding for the A10-specificity had not been isolated. Moreover, the primers which had been previously used to amplify the pBoLA-1 cDNA might also work in selective amplification of A10 cDNA. This is because the 3'-UT primer had 15bp of sequence which was not present in either of A11 genomic clones 19.1 and 4.2. Thus, using this oligo as the 3' primer for PCR amplification, it was likely to selectively amplify A10-like sequences from the cDNA. In order to express and characterise these class I sequences, a cDNA approach was adopted for the cloning of A10-encoding gene from this haplotype into pBoLA-21. Total RNA prepared from a *Theileria annulata*-infected cell line derived from animal 10769 serotype was reverse- transcribed to produce a cDNA template as described in section 2.9.3. 10 μ l of this cDNA was used for the PCR amplification

Fig. 7.1 FACS analysis of the pBoLA-30 transfected cell line with IL-A88. The Ltk⁻ cells were labelled with an isotype-matched negative control antibody (IL-A12). After washing, Log cell fluorescence was plotted against cell number for both the negative control mAb and for the class I specific mAb (red curve).



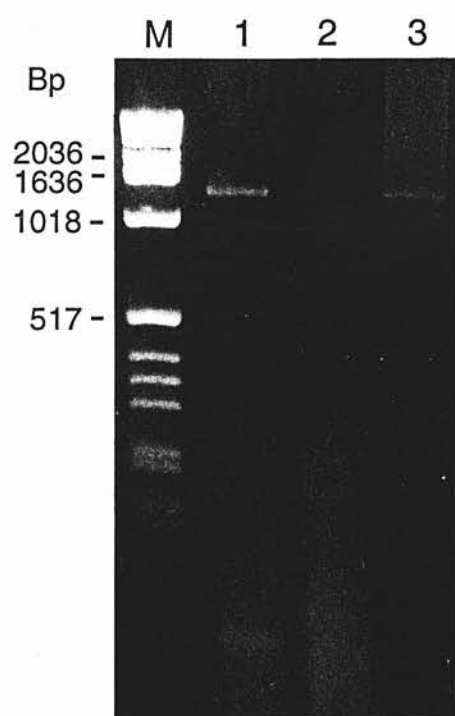
of class I sequences using the exon 2 forward primer 2406 and the reverse primer M0700. PCR conditions for amplification were the same as those used to amplify the pBoLA-1 cDNA section 7.3. A control PCR reaction was also done using the exon 7 forward primer 773 and the 3'-UT reverse primer M0700 to assess the quality of the cDNA used for amplification. A single band of the expected size (approximately 1200 bp) was obtained for the exon 2- 3'-UT cDNA (Fig 7.2) whereas the control reaction with 773 and M0700 gave a 200bp product. The yield of the 1.2kb PCR amplified cDNA appeared to be much less than 200bp amplified control from the intensity of the ethidium bromide stained bands. In order to subclone the 1.2kb product into the TA vector, the amplified products from three reactions were pooled and precipitated with ethanol to concentrate the DNA as described in section 2.6.4. After ethanol precipitation the PCR product DNA was resuspended in 10 μ l of dH₂O, 1 μ l of which was used for TA cloning.

The recombinant clones were screened by colony PCR using exon 2-specific primers as described in section 6.4.1. Three TA clones from 80 transformants gave an exon 2 product of 270 bp and were further examined for the size of the insert fragment. The size of insert was determined by *Eco* RI digestion of plasmid minipreps. One clone contained an insert of 1.2kb which was of the right size, while the other two clones carried shorter inserts, suggesting they were truncated cDNA products. The plasmid DNA from the TA clone with full-length insert was digested with *Eag* I for subcloning the 1.2kb insert fragment into pBoLA-21. Unfortunately, when the plasmid DNA from this clone was digested with *Eag* I to release the 1.2kb cDNA, two insert bands were observed (750 and 450 bp), indicating the presence of an additional *Eag* I site within the insert. In order to subclone the 1.2kb cDNA fragment into pBoLA-21 a different strategy was adopted.

Fig. 7.2 RT-PCR amplification of a 1.2kb fragment using primers 2406 and M0700 from crude RNA prepared from *T. annulata* infected cell line derived from animal 10769.

10% of the PCR products from three independent RT-PCR reactions were analysed by gel electrophoresis on 0.75% agarose gel.

Lane 1 and 3 show a 1.2kb amplified product whereas in Lane 2 no amplification was observed. The molecular weight markers (1kb ladder) are indicated by M.



To obtain a 1.2kb exon 2- 3'-UT cDNA insert fragment from the TA clone, a partial *Eag* I digestion approach was used. Aliquots of plasmid DNA (1.0 μ g) each were incubated with two-fold serial dilutions of *Eag* I restriction enzyme in the manufacturer's buffer at 37°C for 30 minutes. The reaction was then stopped immediately by adding 5 μ l of stop buffer. Two units per μ g were found to give a partial digestion pattern of the plasmid DNA. From the partial digestion products, the complete 1.2kb cDNA fragment was gel-purified for subcloning into *Eag* I-cut pBoLA-21. A clone with the correct orientation of the reconstructed cDNA was isolated by *Sal* I-*Eag* I double digestion and called pBoLA-10. pBoLA-10 was then transfected to investigate the expression of this cDNA clone and to characterise the expressed product.

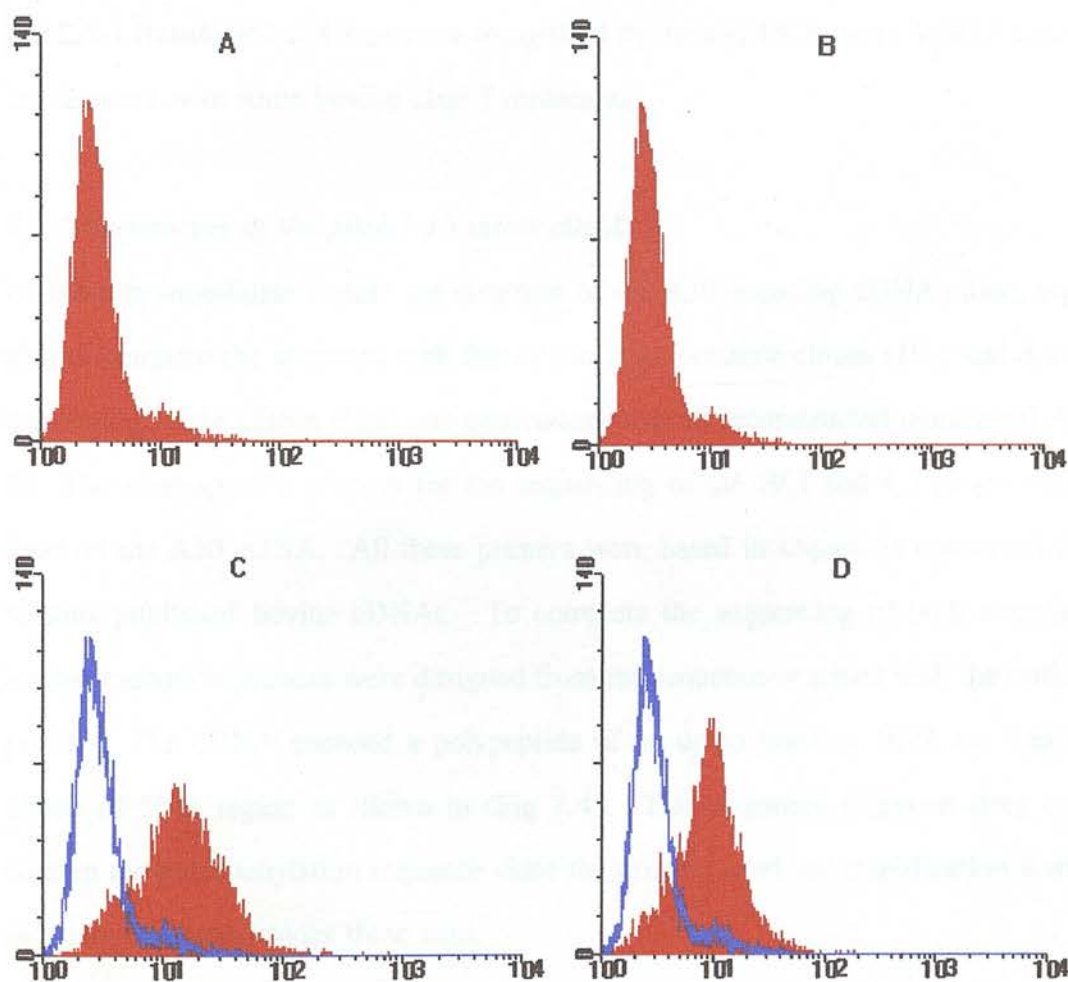
7.7 Characterisation of the pBoLA-10 transfected cell line

Flow cytometry of the primary pBoLA-10 transfectants derived from clone with IL-A88 showed a small population of cells expressing bovine class I molecules. When these were subjected to MACS sorting an increase in the fluorescence of the main body of the class I transfected cells was observed relative to the Ltk⁻ control (Fig 7.3). However, the fluorescence intensity of the positive cells revealed a lower level of expression on this cDNA transfected cell line. Further characterisation of this transfected cell line was done with different A10-specific mAbs.

7.7.1 FACS analysis with A10-specific mAbs

Three A10-specific mAbs (IL-A7, IL-A10 and IL-A34) were used to determine whether the product expressed by the pBoLA-10 transfected cell line had A10 specificity. Only IL-A34 recognised the product expressed on the surface of transfected cells (Fig 7.3). This suggested that transfected cell line probably encoded

Fig. 7.3 FACS analysis of the pBoLA-10 transfected cell line with (A) BoLA-A10 specific mAb IL-A7, (B) BoLA-A10 specific mAb IL-A10, (C) a pan-class I mAb (IL-A88), and the BoLA-A10 specific mAbs and (D) BoLA-A10 specific mAb IL-A34. Cells were labelled with the class I-specific primary antibody, followed by the appropriate fluorescent secondary antibody, and were analysed as described in Fig. 7.1



a component of the BoLA-A10 haplotype. However, the fluorescence intensity of the transfectant stained with IL-A34 mAb was lower than that of staining IL-A88 staining, possibly reflecting a lower affinity of this mAb for the expressed product encoded by this cDNA clone. In a parallel experiment, the pBoLA-30 transfectant cells were also analyzed using the same A10-specific mAbs. None of A10-specific mAbs reacted with the pBoLA-1 transfectant. Interestingly, neither the A10 or pBoLA-1 transfected cell lines were recognised by the anti-HLA mAb W6/32 which cross-reacts with some bovine class I molecules.

7.8.2 Sequencing of the pBoLA-10 insert cDNA

In order to investigate further the structure of the A10-encoding cDNA clone, and also to compare the sequence with that of two A11 genomic clones (19.1 and 4.2), sequencing of the cDNA clone was undertaken from its reconstructed clone pBoLA-10. The exon-specific primers for the sequencing of the 19.1 and 4.2 genes were used on the A10 cDNA. All these primers were based in sequences conserved in various published bovine cDNAs. To complete the sequencing of both strands, further extension primers were designed from the sequence obtained with the initial primers. The cDNA encoded a polypeptide of an up to position 1019 and had a 150bp of 3'-ut region as shown in (Fig 7.4). This sequence fragment does not contain the polyadenylation sequence since the primers used for amplification were based at positions inside these sites.

7.8.3 Features of the pBoLA-10 encoded class I molecule

The predicted amino acid sequence of this BoLA class I cDNA has the features expected of functional antigen-presenting glycoproteins. It has the four conserved cysteine residues at positions 101, 164, 203 and 259 in the α_2 and α_3 domains, for

Fig. 7.4 The nucleotide sequence of the class I gene from the cDNA clone pBoLA-10. The translated amino acids sequences of the exons are given in *three-letter code* above the DNA sequence, and the position of the start of each domain is shown above protein sequence.

α_1 domain
 GlySerHisSerLeuArgTyrPheLeuThrAlaValSerArgProGlyPheGlyGluProArgTyrLeuGluValGlyTyrValAspThrGlnPheValArgPhe
 GGCTCCCACTCGCTGAGGTATTTCTCACCGCGGTGTCCCGGCCGCTTCGGGGAGGCCCGGTACTTGGAAGTCGGCTACGTGGACGACACGAGTTCTGCGGTTTC
 120

AspSerValAlaProAsnProArgMetGluProArgAlaArgTyrValGluGlnGlyProGluTyrTyrPaspGlnGluThrArgLysAlaLysGlyAsnAlaGln
 GACAGCGTCGCCCCGGAATCCGAGGATGGAGCCGCGGGCGCGGTGGTGAGCAGAGGGGCCGAGTATTGGATCAGAGACCGGAAAGGCCAAGGGCAACGACAA
 240

α_2 domain
 PhePheArgValSerLeuAsnAsnLeuArgGlyTyrTyrAsnGlnSerGluAlaGlySerHisThrLeuGlnLeuMetSerGlyCysTyrValGlyProAspGlyArgL
 TTTTTCGAGTGAGCCTGAACAACCTGCGCGGCTACTACAACAGAGCGAGGCCGGGTCTCACACCTCCAGCTGATGTCCGGCTGCTACGTGGGCCCGGACGGCGTC
 360

euArgArgGlyPheMetGlnPheGlyTyrAspGlyArgAspTyrLeuAlaLeuAsnGluAspLeuArgSerTrpThrAlaValGluThrValAlaGlnIleSerLysAr
 TCCGCCGCGGGTTCAATGCAGTTGGCTACGACGCGCAGAGATTACTCGCCCTGAACGAGACCTGCGCTCTTGACC CGCGGTGGAGACGCTGGCTCAGATCTCCAAACG
 480

glyMetGluAlaAlaGlyGluAlaGluValGlnArgAsnTyrLeuGluGlyArgCysValGluTrpLeuArgArgTyrLeuGluAsnGlyLysAspThrLeuLeuArg
 CAAGATGGAGGCGCGCGGTGAAGCTGAGGTACAGAGGAACACTACCTGGAGGGCCGGTGGAGTGCTCCGACAGATACCTGGAGAACGGGAGACACGCGCTGCGC
 600

α_3 domain
 AlaAspProProLysAlaHisValThrHisHisProIleSerAspArgGluValThrLeuArgCysTrpAlaLeuGlyPheTyrProGluGlnIleSerLeuThrTrpG
 GCAGACCTCCAAAGGCACATGTGACGCATCACCCCATCTCTGACCGTGAAGTCAACCTGAGGTGTGGGCCCTGGGCTTCTACCTGAGGAGATCTCACTGACCTGGC
 720

LInArgAsnGlyGluAspGlnThrGlnAspMetGluLeuValGluThrArgProSerGlyAspGlyAsnPheGlnLysTrpValAlaLeuValProSerGlyGluGln
 AGCGCAATGGGAGGACACAGACGCAGACATGTGAGACTTGTGAGACACAGGCCCTTCAGGGGACGGAACCTCCAGAGTGGGTGGCCCTGTGTGCTTCAGAGAGGA
 840

uGlnArgTyrThrCysArgValGlnHisGluGlyLeuGlnGluProLeuThrLeuArgTyrGluProProGlnProSerPheLeuThrMetGlyIleIleValGlyLeu
 GCAGAGATACACGTGCGAGTGACAGCAGAGGGGCTTCAGAGGACCCCTCAACCTGAGATGGAACTCTCAGCCCTCTTCACCATGGGCATCATTTGGCCCTG
 960

cytoplasmic domain
 ValLeuLeuValValThrGlyAlaValAlaGlyValValValCysMetLysLysArgSerGlyLysArgGlyThrTyrIleGlnAlaSerSerSerAspSera
 GTTCTCTCGTGGTCACTGGAGCTGTGGTGGCTGGAGTTGTGTCTGTGATGAAGACGCTCAGGTGAAGGACCTTATATTCAGGCTTCAAGCAGATGACAGTG
 1080

laGlnGlySerAspValSerLeuThrValProLysValEnd
 CCCAGGGCTCTGATGTCTCTCAACGGTTCTCTAAAGTGTA
 1200

Primer	Location	Sequence
770	exon 2 forward	5' GCTCCCACTCTGAGGTATT 3'
771	exon 2 reverse	5' CTCGCTCTGGTTA/GTAGTAGCC 3'
N0700	exon 2 forward	5' GAGGGGCGYSGAGTATTGG 3'
N0701	exon 2 reverse	5' CCAATACTCSRGCCCCCTC 3'
1595	exon 3 forward	5' GGCTGCG/TACGTGGGGCC 3'
1596	exon 3 reverse	5' TCCAGGTATCTGCGGAGC 3'
N0699	exon 3 reverse	5' CCGCGGTCCAGGAGYGAC 3'
N0698	exon 3 forward	5' TGCRCCTCCTGGACCGCGG 3'
1597	exon 4 forward	5' CCTCCAAAGGCACATGT 3'
8442	exon 4 reverse	5' CTCCACAAGCTCCATGTC 3'
1599	exon 5 reverse	5' CCTCAGA/CCCTCCTTCCTC 3'
1600	exon 7 reverse	5' AGACACATCAGAGCCC 3'
773	exon 7 forward	5' GGCTCTGATGTGTCTCTCACG 3'
772	exon 8-3'-ut rev	5' GATGMAGCATCACTCAGTCCC 3'
M0700	3'-ut reverse	5'GGGGGCGGCCGTGGGGARGGGCAG CAGTGGG 3'

Table 7.1 : Sequencing primers used for pBoLA-10 and their location on A10 gene.

intra-domain disulphide bond formation. There is a single conserved site for N-linked glycosylation at position 86 in α_1 domain and there is also a conserved phosphorylation site at serine 333 in the cytoplasmic domain (Guild and Strominger, 1984). The transmembrane region of this cDNA class I sequence contained 37 amino acids as seen in published BoLA class I cDNA A10, pBoLA-1 and BL3-6 (Bensaid et al. 1991; Brown et al. 1989; Ennis et al. 1988).

7.9 Discussion

The PCR amplification system to subclone coding sequences from genomic and cDNA into the expression cassette (pBoLA-21) provides a rapid way of isolating and characterising functional bovine class I genes. The described approach could also be very useful for the expression of bovine class I genomic or published cDNA clones which lack exon1 and /or 3'-untranslated regions (Garber et al. 1993, 1994). Furthermore, different 3'-end primers can be used to select all class I genes, or genes potentially from different loci. As more bovine class I sequence information becomes available, it may be possible to identify locus-specific sequences in the untranslated regions of class I genes. Using such locus-specific regions as primers, it would be easy to selectively amplify, clone and express the different bovine class I genes using this system. Selective amplification of class I cDNA sequences for HLA-C and H-2D or H-2L by using locus specific primers have also been described previously by (Zemmour and Parham, 1992; Cai and Pease, 1990). Locus specific substitutions have also been found in 3'-untranslated regions of HLA class I genes (Koller et al. 1984; Strachan et al. 1984).

Only a single full length 1.2kb cDNA clone was obtained using the 3'-end primer M0700. This does not however, suggest that there was only one A10 like gene present in this haplotype. The failure to isolate other A10 -like sequences appeared to be due to the poor efficiency of TA cloning of the PCR amplified cDNA. It was not surprising to find two out of three cDNA clones obtained were truncated, which probably reflects the quality of cDNA template due to inefficient reverse transcription and also non-specificity of the PCR due to the size of the annealing regions on both 5' and 3' primers. Although the presence of additional *Eag* I site in the insert made the cloning exercise difficult, but it supported the evidence of

selective amplification of A10 cDNA with 3'-end primer as both the 19.1 and 4.2 sequences lacked *Eag* I site in the 3.2kb insert fragment.

The characterisation of the pBoLA-10 transfected cell line with three A10-specific mAbs revealed that only IL-A34 recognised the expressed product. Further analysis of pBoLA-10 transfectants revealed that the expressed product from A10 cDNA was not seen by the anti HLA class I mAb W6/32. Biochemical studies by Joosten et al. (1992) showed that a class I molecule associated with A10 specificity was immunoprecipitated by IL-A34 from the A10 haplotype. This molecule was not recognised with W6/32 in the same experiment. The FACS analysis data from the A10 transfected cell line when compared to the findings of Joosten and co-workers (1992), suggests that A10 cDNA isolated here might encode the molecule which was immunoprecipitated by IL-A34 in their experiments. Since PBM from this animal were recognised by IL-A7 and IL-A10. So it is expected that one or more other genes encoding components of the A10 haplotype are also expressed in this animal.

Chapter 8

ANALYSIS OF BoLA CLASS I SEQUENCES

8.1 Introduction

The major histocompatibility complex has been the subject of intense investigation due to the high polymorphism of its genes and its central role in the immune system. Between different species and even between strains of mice, the number of MHC genes varies and this is especially true for the class I genes (Rogers, 1985); eg. more than 60 have been found in rat compared to as few as six in pigs and perhaps only one in Syrian hamster and Cheetah (Kindt and Singer, 1987; O'Brien et al. 1985). Although the number of class I genes in man may be as high as 30 (Driesel et al. 1985), there are only three functional class I genes (classical): in humans HLA-A, B, C; and in mice H-2K, D, L. Amongst these multiple genes however, one can discern many features shared by classical class I molecules of all species, presumably because of selection through antigen presentation. Thus, residues important for maintaining the structure of the heavy chain and its interactions with β_2m , TCR, CD8 and antigenic peptides are conserved in all classical class I molecules. Despite the potential advantage of expression of multiple MHC genes, the number of class I genes actually used in antigen presentation appears to be small, usually one to three, and others factors relating to the establishment of immunological self-tolerance may act to limit this number (Matzinger et al. 1984).

Most class I genes are composed of eight exons separated by seven introns; exceptions include the human *p12.4* pseudogene and mouse *Q10* and *TL* genes (Fisher et al. 1985). The corresponding introns and exons of class I genes are well conserved (Jordan, 1985), e.g. the lowest homologies found among five HLA genes were 79% for exon 8 and 64% for intron 7 (Sodoyer et al. 1985). This is in

contrast to comparisons between species (mouse and man) which have shown that, while the exons share a substantial degree of homology (75-85%), the introns are essentially non-homologous. The length of the introns with exception of introns 3 and 5 is similar between class I genes, whether comparisons are being made between alleles, loci or species. Intron 3 is variable in length amongst the mouse class I genes, and is longer than its human counterpart, whereas intron 5 is longer in humans (Lew et al. 1986).

Understanding the polymorphisms of class I genes requires comparison of the allelic sequences. This simple proposition is complicated by uncertainty as to the total number of alleles and the presence of many different class I genes and pseudogenes in the human and murine genomes. Analysis of HLA-A,B,C sequences has shown that within alleles of a locus, the 5' part of the gene, encompassing exons 1 to 3, is highly variable and shows few locus-specific substitutions; while the 3' part of the gene, exons 4 to 8, is relatively conserved and contains many locus-specific nucleotides (Parham et al. 1989). Most of the substitutions in this region are seen in the transmembrane and cytoplasmic domain-encoding exons and in the 3' untranslated region (Coppin et al. 1985; Koller et al. 1984). However, the degree of allelic variation differs between HLA loci. The HLA-B alleles are highly variable in the 5' exons and homogenous in the 3' exons. In contrast, HLA-A alleles show less variability in the 5' exons and less homogeneity in the 3' exons.

In cattle, polymorphism at the BoLA class I loci has been detected primarily by serological methods (Bernoco et al. 1991; Bull et al. 1989). This has enabled the identification of 52 serological specificities, most of which behave as alleles of single highly polymorphic locus (Davies et al. 1994). However, there is limited serological

evidence for a second locus by reagents (Stear et al. 1982). When biochemical (Al-Murrani et al. 1993, 1994) and molecular techniques are employed, there is good evidence for more than one class I locus in cattle (Ennis et al. 1988; Brown et al. 1989; Toye et al. 1990; Bensaid et al. 1991; Ellis et al. 1992; Garber et al. 1993, 1994). These loci are apparently in linkage disequilibrium (Al-Murrani et al. 1993), making them difficult to distinguish by conventional methods. However, based on the number of the amino acids in transmembrane region, the published class I cDNA sequences suggest the presence of three loci. SDS-PAGE, ID-IEF and peptide mapping analysis of charge heterogeneity of bovine class I molecules have confirmed at least three BoLA class I loci are expressed at the protein level (Al-Murrani et al. 1994).

In this work three BoLA class I sequences (pBoLA-19, pBoLA-4 and pBoLA-10) were isolated from a single animal, heterozygous for the BoLA serological specificities BoLA-A10 and BoLA-A11. The sequences were derived from two genomic and one cDNA class I clone. All three clones expressed a detectable bovine class I product on mouse L cells in transfection experiments, which suggest that they represent functional class I genes. The expressed products from the genomic class I clones pBoLA-19 and pBoLA-4 were typed as A11 by serological, biochemical and cellular methods as described in section 4.3. In addition, a cDNA clone, isolated by PCR amplification using a selective primer based in the 3'-ut, expressed A10 specificity. In order to determine whether these sequences were alleles of same locus or from different loci, the sequences were compared with each other and with all of the published BoLA cDNA sequences.

8.2 Comparison of pBoLA-19 and pBoLA-4 sequences

In order to determine whether the A11-encoding pBoLA-19 and pBoLA-4 sequences were from different loci or were alleles of the same locus, comparative analysis of the sequences of the two genomic clones was done. The sequences exhibit 92.8% nucleotide identity in the coding region and 88% identity at the amino acid level. The length of the transmembrane region of both the sequences was 35 amino acids. However, the two sequences appeared to be more diverged in the 3' part of the gene particularly in the transmembrane and cytoplasmic domains. Both these regions of have been suggested to contain locus-specific residues (Davidson et al. 1985). This comparison sequence suggested that these two sequences were likely to be different genes rather than alleles of the same locus. Interestingly, in spite of the differences between the two sequences, the expressed products from these class I genes were indistinguishable by IEF analysis. To investigate whether similarity in isoelectric point of the products of pBoLA-19 and pBoLA-4 was in accord with the predicted amino acid sequences of the proteins, the pI of the pBoLA-19 and pBoLA-4 encoded products was calculated. However, the leader peptide was not included for the calculation of pI.

8.2.1 Determination of isoelectric point of pBoLA-19 and pBoLA-4.

The PC/GENE program ISOELECTRIC (Intelligenetics) was used to determine the isoelectric point of the two gene products by computer. This program calculates the isoelectric point of a protein from its amino acid sequence assuming that no isoelectrostatic interactions occur that perturb ionisation. ISOELECTRIC then plots the total positive and negative charges and the net charge of the protein as a function of pH. When the predicted amino acid sequences from the pBoLA-19 and pBoLA-4 were compared using this program the values were found to be very similar ie 5.26

for pBoLA-19 and 5.23 for pBoLA-4. This difference of 0.03 pI units between these two class I molecules would not be differentiated on IEF gel and this is in accord with the observation that the class I molecules from these two genes migrated to same position in IEF analysis. Many HLA antigens have indistinguishable IEF patterns ie HLA-A3 and HLA-B7, B8 and B27.5 (Baur and Danilovs, 1980; Guttridge and Klouda, 1989). These results indicate that different locus products can have very similar pI values and that the pBoLA-19 and pBoLA-4 sequences represent different genes encoding components of the A11 haplotype.

8.3 Comparison of three BoLA class I sequences with other published bovine class I cDNA

Recent molecular cloning studies involving BoLA class I genes have resulted in the isolation of a number of bovine class I sequences by various workers. The identifiers assigned to the sequences by the EMBL database, and names of corresponding clones described in the publication are given in Table 8.1.

Table 8.1

Sequence	Clone	Reference
Btbolaa	BL3-6	Ennis et al. 1988
Btbolab	BL3-7	<i>do</i>
Btmhla	pBoLA-1	Brown et al. 1989
Bimhawa	Aw10	Bensaid et al. 1991
Bimhbka	Kn104	<i>do</i>
Btmhcia	BSA	Garber et al. 1993
Btmhcib	BSC	<i>do</i>
Btmhcic	BSF	<i>do</i>
Btmhcid	BSN	<i>do</i>
Bt187	BSX	Garber et al. 1994
Btla A11	pBoLA-19	Sawhney et al. 1995
	pBoLA-4	unpublished
	pBoLA-10	unpublished

This sequence data appears to demonstrate the presence of at least three MHC class I loci in cattle (Garber et al. 1994). But as yet the total number of cell-surface expressed class I genes in any haplotype has not been established. Ennis and co-workers were first to report bovine cDNA clones (BL3-6 and BL3-7) which were suggested to be products of different loci. This finding was based upon differences

in the 3'ut regions and in the length of the transmembrane regions. In another report (Bensaid et al. 1991), two cDNA clones were isolated from a homozygous animal expressing the BoLA types W10 and KN104, and these clones showed divergence at both ends of the gene. When the 3'untranslated regions of W10 and KN104 clones were used as probes in Southern blots only a single fragment was revealed in each digested sample, showing these probes had locus specificity. Further evidence for the presence of at least two class I BoLA loci came from the use of the polymerase chain reaction to amplify fragments of expressed bovine MHC class I genes from cDNA (Ellis et al. 1992). The results suggested the presence of four alleles representing the products of two class I loci. Ellis and co-workers (1992) also found the presence of alternatively spliced mRNA which results in the removal of exon 7, predicting a truncated molecules with the cytoplasmic tail 16 amino acids shorter than usual. Garber et al. (1993, 1994) isolated six different class I cDNAs from a single heterozygous animal which is strong evidence of three class I loci in the cattle. However in none of these studies was the transcription of the genes related to surface expression of class I products.

8.3.1 *Three new BoLA class I sequences encode functional antigen presenting molecule.*

Comparison of the predicted amino acid sequences of pBoLA-19, pBoLA-10 and pBoLA-4 with those of all published BoLA class I cDNAs demonstrated that three genes have features of functional antigen-presenting molecules. The various functionally-important amino acid residues have been marked on Fig 8.1. The only notable difference is that pBoLA-4 sequence has arginine in place of leucine 334 in the consensus sequence for phosphorylation (Ser-Asp/Glu-Xaa-Ser(P)-Leu). This sites is conserved across species, but has not been shown to be of functional

significance (Guild and Strominger, 1984). However, since the phosphorylated residue (Ser 333) is conserved, it is not clear whether this substitution would affect its phosphorylation.

8.4 Sequence alignment

The alignments presented in this section were generated using the program PILEUP from the GCG package, version 7.3. This program creates a multiple sequence alignment using progressive pairwise alignments. The differences between the various pairwise sequences alignments may then be calculated by the program DISTANCES from the PILEUP output. On the basis of these genetic distances a phylogenetic tree was generated using the full length amino acid sequences, which split the sequences into two groups as shown earlier by Garber et al. (1993). Two of the cDNAs (pBoLA-4 and KN104), however, were no more closely related to either of the two branches (Fig 8.2). These data thus suggested the presence of at least four BoLA class I loci, represented by five cDNAs in cluster A (BL3-6, BSA, pBoLA-1, A10 and pBoLA-10), four cDNAs in cluster B (BL3-7, pBoLA-19, BSN and BSF), and a single cDNA each in clusters C (KN104) and E (pBoLA-4). The clustering in the alignment suggested that genetic distance between the A11 sequences (pBoLA-19 and pBoLA-4) was sufficiently large which makes them more likely to be different genes rather than alleles. These sequences shared 88% similarity at the nucleotide level and the amino acid differences in the transmembrane and cytoplasmic regions support the suggestion from alignment data that these two sequences come from different loci.

The pBoLA-10 sequence was found on the same branch as the A10 cDNA. These sequences shared 97% identity at the amino acid level. This suggests that pBoLA-10

Fig. 8.1 Multiple alignment of the predicted amino acid sequences of 12 BoLA class I sequences. Dashes indicate residues identical to the consensus. Asterisks indicated amino acids whose side chains are directed into the peptide binding site. A conserved site (NQS) for N linked glycosylation at positions 86-88, a conserved site for phosphorylation (S[DorE] XS{PO4} L) at positions 330-334, and sites for intradomain disulphide bond formation (cysteines 101, 164, 203 and 259) are all *underlined*. Amino acids in *bold* show unique substitution in the pBoLA-4 sequence in the transmembrane and cytoplasmic domains.

	α_1 domain				50
BL3-7	-----y-	g-----	--ia-----	-----	---e---vp
pBoLA-19	-----y-	-----	--ia-----	---t-----	---d---vp
BSF	-----	-----	--ia-----	---t-----	---e---p
BSN	-----l-	-----	--ii-----	-----nt	-----
pBoLA-4	-----l-	r---d---	--l-----n	-----	r-----
BL3-6	-----	-----	--l-----	---q-----	-----
BSA	-----	-----	--l-----	---q-----	-----
Aw10	-----	-----	--l-----	-----	-----
pBoLA-10	-----l-	-----	--l-----	-----v-	-----
pBoLA-1	-----	-----	--l-----	-----	-----
BSC	-----y-	-----	--is-----n	-e-----	-d-e---v-
KN104	-----l-	-----vr--	--ii-----	l-----	gd-v----
Consensus	GSLSLRYFST	AVSRPGLGEP	R--EVGYVDD	TQFVRFDSDA	PNPRMEPRAR
	* * *		* * *		

	51	α_2 domain				100
BL3-7	-m-----	----iy-d-	--i---d---	-----t	---ni-a---	
pBoLA-19	-m-----	----iy-d-	--i-a---	al-----	-----e-	
BSF	-m---l---	----iy-d-	-----y---	-----	-----s-	
BSN	---k-----	-e-ns-e-	---a---	al-----	---v-e---	
pBoLA-4	-----	-q-ns-ga	-----d---	-----	---i-a---	
BL3-6	-----	---n-gn	-s-----	-----	-----s-	
BSA	-----	---n-gn	-s-----	-----	-----s-	
Aw10	-----	-e-qr-gn	-f--s--n	-----	-----s-	
pBoLA-10	-----	-qe-k-gn	-f--s--n	-----	---l-s-	
pBoLA-1	-----	-qe-k-g-	---a---i	al-----	-----	
BSC	-m---l---	----iy-d-	---d---	-----	-----e-	
KN104	---k-----	-ee-r-dh	-f--rg--a	-----	-----e-	
Consensus	WVEQEGPEYW	DRNTR-AK-T	AQTFRVNLNT	LRGYYNQSEEA	GSHTLQWMYG	
	***	*****	*****	* * * *	* * *	

	101					150
BL3-7	-----	---w-----	-----	-----	-----	
pBoLA-19	-y-----	l-----a---	-----	-----	-----	
BSF	-----g---	-----	---q---	-----	-----	
BSN	-----	---da---	-----	-----	-----	
pBoLA-4	-----	---r-da---	-----	-----	---e---	
BL3-6	-----a-r	-----	---l-----	---g-e---	-----	
BSA	-----r	-----	---l-----	---g-e---	-----	
Aw10	-y-----pp	-----	---l-----	---v-m---	-s--m---	
pBoLA-10	-y-----r	-----	---l-----	---v-v---	-s--m---	
pBoLA-1	-----r	-----	-----	-----	-----	
BSC	-----g-e---	---l-----	-----	-----	h-s--v---	
KN104	-y-----	---e-----	---g---	h-----	-s-----	
Consensus	<u>C</u> DVGPDGRLL	RGFMQFGYDG	RDYIALNEDL	RSWTAADTAA	QITKRKWEAA	
		* *			* * * *	

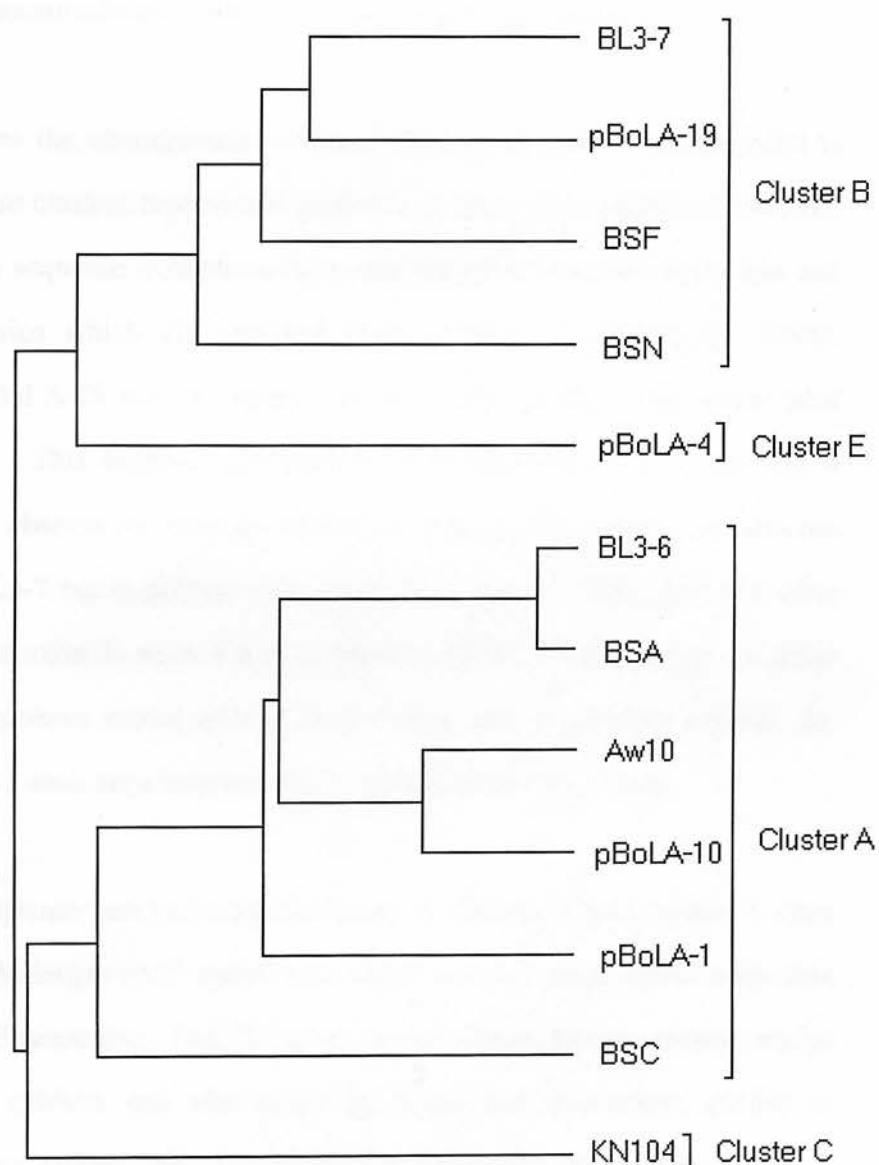
	α_3 domain				200
BL3-7	-a--tw---	---e-----	-----	-----	---s-----
pBoLA-19	---r-----	---r--g---	-----	-----	-----
BSF	dn--se---	---e-----	-----	-----	-----
BSN	---tw---	---r--g---	-----a---	-----	---s-----
pBoLA-4	-a--ge---	---t-----	---a---	-----	-----
BL3-6	-y-----	---e-----	-----	-----	---g-----
BSA	-y-----	---e-----	-----	---a-----	-----
Aw10	-----	---r-----	-----	-----	r---g---
pBoLA-10	-----	---r-----	-----	-----	-----
pBoLA-1	---r-----	---t-----	---t-----	-----	---s-gh---
BSC	---rf---	---r--g---	---a---	-----a	-----
KN104	---st-fy---	---e-----	-----	-----	-----
Consensus	GEAEVQRNYL	EG-CVEWLRR	YLENGKDTLL	RA DPPKAHVT	HHPISDREVT
	** * * * *	*** * * *	*		

	201				250
BL3-7	-----	-----	-----	-----	-----
pBoLA-19	-----	-----h-	-----y-	-----	-----
BSF	-----	-----n	-----y-	-----	-----
BSN	-----	-----	-----	-----	-----a-
pBoLA-4	-----	-----	-----	-----s	-----
BL3-6	-----	-----h-	-----	-----	-----
BSA	-----	-----h-	-----	-----	-----
Aw10	-----	-----n	-----	-----n	-----l-
pBoLA-10	-----	-----n	-----	-----n	-----v-
pBoAL-1	-----	-----n	-----	-----n	-----
BSC	-----	-----h-	-----	-----	-----
KN104	-----	-----h-	-----	-----	-----v-
Consensus	LRCWALGFYP	EEISLTWQRD	GEDQTQDMEL	VETRPSGDGT	FQKWAALVVP

		transmembrane domain				
	251					300
BL3-7	-----	-----	-----t-	-----i-	-----	-----l-
pBoLA-19	-----	-----	-----t-	-----	-----	-----
BSF	p-----	-----	-----t-	-----i-	-----	-----l-
BSN	-----	-----	-----t-	-----	-----	-----
pBoLA-4	-----	-----k-	-----i-	-----vpi-	-----	-----l-
BL3-6	-----	-----	-----	-----	-----	-----
BSA	-----	-----	-----	-----	-----	-----
Aw10	-----k---q	-----	-----k-	-----	-----	-----
pBoLA-10	-----	-----	-----	-----	-----	-----
pBoLA-1	-----k---	-----	-----k-	-----	-----	-----
BSC	-----	-----	-----	-----	-----	-----
KN104	-----	-----	-----t-	-----	-----	-----
Consensus	SGEEQRYTCR	VQHEGLQEPL	TLRW	EPPQPS	FLTMGIIVGL	VLLVVTGAVV

		cytoplasmic domain				
	301					339
BL3-7	--a--wr---	----	ri-t-	-a-----	-----	-----
pBoLA-19	--a--wr---	----	ri-t-	-a-----	-----	-----
BSF	--a--wr---	----	ri-t-	-a---t---	-----a	-----
BSN	--a--wr---	----	ri-t-	-a---t---	-----	-----
pBoLA-4	--a--wr--c-	----	qt-t-	-a---d---	-----r-	-----
BL3-6	-----c---	----	n-	-----	-----	-----
BSA	-----c---	----	n-	--g-----	-----	-----
Aw10	-----c---	----	r-t-	-----	-----	-----
pBoLA-10	-----c---	----	r-t-	-----	-----	-----
pBoLA-1	-----c---	----	r-t-	-----	-----	-----
BSC	-----c---	----	n-	--g-----	-----	-----
KN104	--f--w----	----	n-	-----a-r-	-----	-----
Consensus	AGVVI-MKKR S	GEKGG-YIQ	ASSSDSAQGS	DVSLTVPKV		

Fig. 8.2 Phylogenetic tree of BoLA class I sequences based upon the alignment of the complete amino acid sequences in figure 8.1, constructed from the PILEUP output. The tree was rooted by including human sequences in the alignment. Cluster (A-E) are named in accord with Garber et al. (1994) and may represent class I loci.



and A10 class I cDNA are alleles from the same locus. These sequences are identical in the transmembrane and cytoplasmic domains. In addition, pBoLA-1 (Btmhla) was always found to be closely associated with both A10-specific sequences in this analysis, which suggests this sequence is also from the same locus. The KN104 sequence formed a distant member of this group because of its unique amino acid substitutions in transmembrane domain it was assigned to cluster C.

In previous studies the characteristics of the cattle A, B, and C cluster cDNAs suggested that these clusters represented products of three classical class I loci; and this was based on sequence differences in exons encoding the transmembrane and cytoplasmic domains which distinguished these clusters (Garber et al. 1993). Analysis of the pBoLA-19 coding sequence showed 97% identity at the amino acid level with BL3-7. This suggests that pBoLA-19 is allelic to BL3-7, and this is supported by the observation that 21 of the 26 amino acid differences between pBoLA-19 and BL3-7 are in polymorphic the exons 2 and 3. There are only three substitutions in the exon 4- exon 8 region between these two sequences. Similar high nucleotide sequence homologies of both coding and non coding regions ie, 94.5% and 97.2%, were seen between HLA-A2 and HLA-A3 alleles.

The pBoLA-10 sequence grouped with the cluster A cDNAs. These cluster A class I sequences had TM length of 37 amino acid which was two more amino acids than the cluster B and E sequences. This difference in the length transmembrane region of BoLA class I cDNAs was also noted by Ennis and co-workers (1988) as supporting evidence of two loci. Locus-specific differences in the length of the transmembrane domain also occurs in humans where HLA-C gene products have one more amino acid than do the HLA-A and HLA-B gene products. This is one of the

regions which contain locus-specific residues which are apparent when comparing alleles (Gussow et al. 1987). These results suggests that pBoLA-10 sequence is a different locus than pBoLA-19 and pBoLA-4. Since pBoLA-19 and pBoLA-4 sequences were also from different loci based on the data in section 8.2.1, all the three sequences isolated from this animal are likely to be potential candidates to represent three different loci.

8.4.1 *Phylogenetic analysis*

Unlike the exons encoding the α_1 and α_2 domains, where high rates of nucleotide substitutions are found in the ABS as a result of the selection (Hughes and Nei, 1988), substitutions that occur in the more conserved regions of MHC class I genes are often locus-specific, and therefore the analysis of such conserved regions may yield information about relationships among MHC class I sequences where there is no clear locus-specific assignment by other methods. In order to make the comparison more locus-specific, and also to include the truncated sequence Bt187 (Garber et al. 1994), the three class I sequences determined here were aligned with the other BoLA class I sequences using only the region between exon 4 and 8. Phylogenetic trees based on these alignments were constructed using a number of methods, all taken from PHYLIP package version 3.5c. In all cases human MHC sequences were used as outgroups to root the trees. The principle method used for analysis was the DNADIST program which estimates the genetic distance based on the nucleotide identity between each pair of sequences. A tree was constructed using NEIGHBOR program based on DNADIST data on the exon 4 -exon 8 DNA sequence alignments (Fig 8.3). There are three main branches, one of which contained only Bt187 and pBoLA-4. Each of these two sequences has numerous unique nucleotide substitutions which appears to prevent them from being more

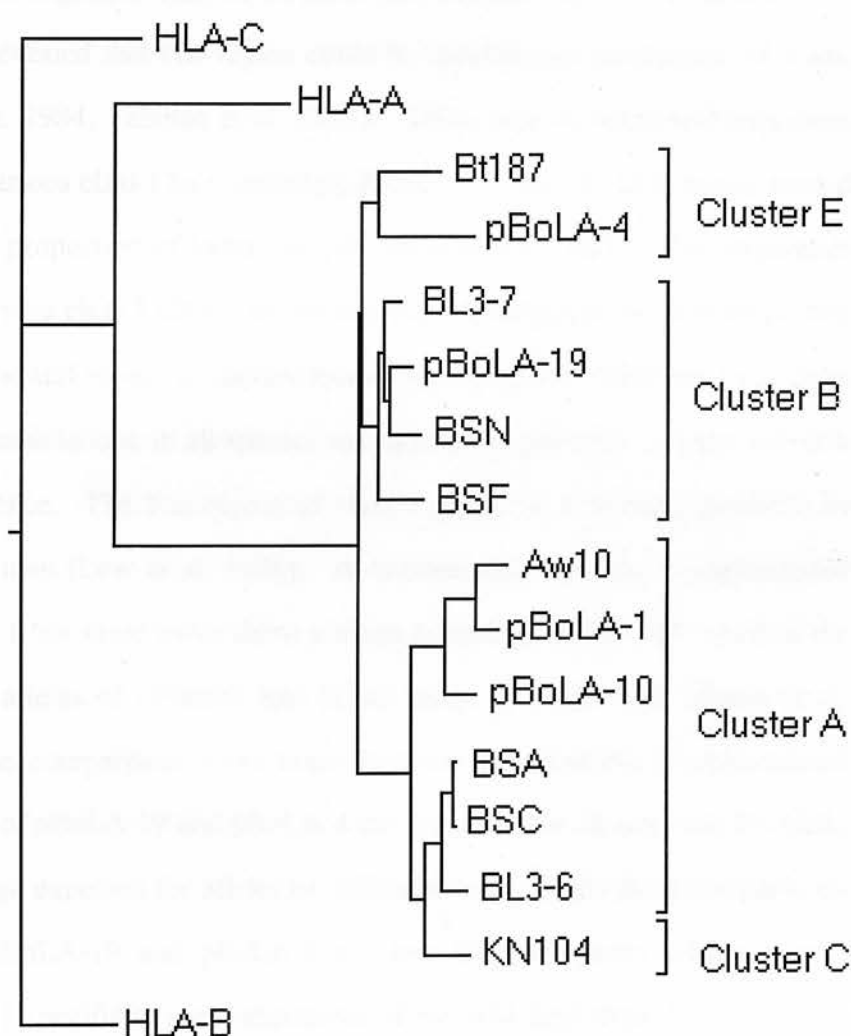
closely grouped with any of the other BoLA cDNAs. These nucleotide differences also contribute to the long branch length that separates Bt187 from pBoLA-4. These two sequences were always grouped together in all of the different methods of analysis used. At the DNA level, Bt187 and pBoLA-4 sequence are 94% identical. As there was no sequence available from the 3'ut region of Bt187, the comparison could only be done with the exon 4-exon 8 region. However, there is no strong evidence from this data that these sequences are from same locus or they are different loci.

The other two branches of this tree (Fig 8.3) resembles with phylogenetic tree constructed by using the exon 2-exon 8 peptide sequences (Fig 8.2). Thus, exclusion of polymorphic exons 2 and exon 3 for the analysis of class I sequences did not make any significant influence on the topology of the tree. However, KN104 was exception as it was always found to be associated with the A10- like sequences (Aw10, pBoLA-10 and pBoLA-1) in all methods of analysis, but based on physical mapping KN104 and Aw10 were shown to be different loci (Bensaid et al. 1991).

8.4.2 *Comparison of the intron sequences of pBoLA-19 and pBoLA-4*

To further determine whether pBoLA-19 and pBoLA-4 sequences are likely to be from different loci, a comparison of the intron sequences was made. The two complete intron sequences between exons 4 and 5 and between exons 7 and 8, were compared from the two genomic sequences pBoLA-19 and pBoLA-4. While the size of the introns between these two genomic clones was generally less well conserved, the comparison of introns 4 and 7 revealed small insertions or deletions. The intron sequences were less conserved than the flanking exons and overall identity of the intron sequences compared was between 88-90%. Since there are no other bovine

Fig. 8.3 Phylogenetic tree of BoLA class I DNA sequences exon encoding α_3 , transmembrane, and cytoplasmic domains of BoLA class I sequences. The tree was constructed using the DNADIST and NEIGHBOR programs within the PHYLIP program package version 3.5 based on sequence alignments like in Fig 8.1



genomic class I sequences available it was difficult to conclude from these results whether this degree of identity in introns was sufficient to assign these two class I sequences to different loci.

8.4.3 *Comparison of the 3'-untranslated region*

It has been suggested that the non-coding and flanking sequences are less well conserved between sequences from different loci compared to alleles from the same locus. Nucleotide sequence analysis of the 3' untranslated regions of mouse class I cDNA clones revealed that this region could be divided into subregions NC1 and NC2 (Kress et al. 1984; Lalanne et al. 1985). These regions contained sequences specific for the various class I loci, including H-2K, D, L but the NC2 region tended to have a greater proportion of locus-specific residues than NC1. The sequences in the 3'-ut of bovine class I cDNA clones are more diverged from each other than the rest of the gene and appear to contain locus-specific motifs. This region is more prone to insertions/deletions in all species and appears to generally contain AT-rich stretches of sequence. The 3'ut region of class I genes has also been shown to be locus specific in man (Lew et al. 1986). In humans and mice the 3'-untranslated regions of alleles from same locus show a mean homology of 94-98% whereas the identity between alleles of different loci in the range of 74 to 95% (Ennis et al. 1988). When the comparisons were made between the available 3'-untranslated region sequences of pBoLA-19 and pBoLA-4 the degree of the identity was 83.42%, clearly in the range expected for alleles of different loci. From these comparisons it appears that pBoLA-19 and pBoLA-4 are two different genes which encode products with A11 specificity are components of the A11 haplotype.

8.5 Discrimination of intralocus and interlocus alleles on the basis of pairwise differences

To further support the above analysis, the pairwise differences between all of the BoLA class I sequences were calculated. For every pairwise combination of sequences, the number of amino acid differences were calculated and their frequency distribution was plotted (Fig 8.4). Calculation of similar pairwise amino acid differences between pairs of HLA-A, B, C sequences generates distinct ranges of values, depending upon whether comparisons are between alleles of the same locus or of different loci. The range of differences for alleles of the same locus is 0 to 21 amino acid substitutions compared to 42 or more for alleles of different loci. (Parham et al. 1995). Pairwise comparison of HLA-B and HLA-C alleles shows fewer differences than when either HLA-B or HLA-C alleles are compared to HLA-A. In the bovine class I sequences this comparison resulted into two major groups as observed from the histogram pattern (Fig 8.4). These groups were represented by pairs of sequences which had scores of 0-8 and 10-22 respectively. All intralocus comparisons are likely to be within the range 0-8 whereas sequences pairings which had differences 10-22 appear to generally come from different loci. Within the 0-8 range the identity between the pairs were found more than 90% which is expected for alleles. This holds true for all the cluster A and B sequence pairs. However, the sequence pairs which fall in the middle range of 10-12 were not clearly separated from the either group but based on the previous analysis they were from different loci. The pairwise differences for these class I sequences are given in table 8.2.

The three sequences KN104, Bt187 and pBoLA-4 when paired with the most of the other sequences showed differences in the range 17-22 suggesting these sequences were distinct from the other BoLA class I sequences and each other. This

Fig. 8.4 Histograms showing the distribution of amino acid differences from the pairwise comparisons of BoLA class I sequences assigned in Fig 8.1. For every pairwise combination of sequences, the number of amino acid differences were computed and their frequency distribution was plotted.

observation is supported from the analysis of the 1000 Genomes Project, which reported that no sequences were found on the reference genome with the exact same amino acid sequence.

The pairwise comparison of protein sequences is a common way to identify differences between sequences expected for protein variants. The number of amino acid differences between two sequences is the difference between the number of amino acids in the two sequences. The number of amino acid differences between two sequences is a measure of the genetic distance between the two sequences. The number of amino acid differences between two sequences is a measure of the genetic distance between the two sequences. The number of amino acid differences between two sequences is a measure of the genetic distance between the two sequences.

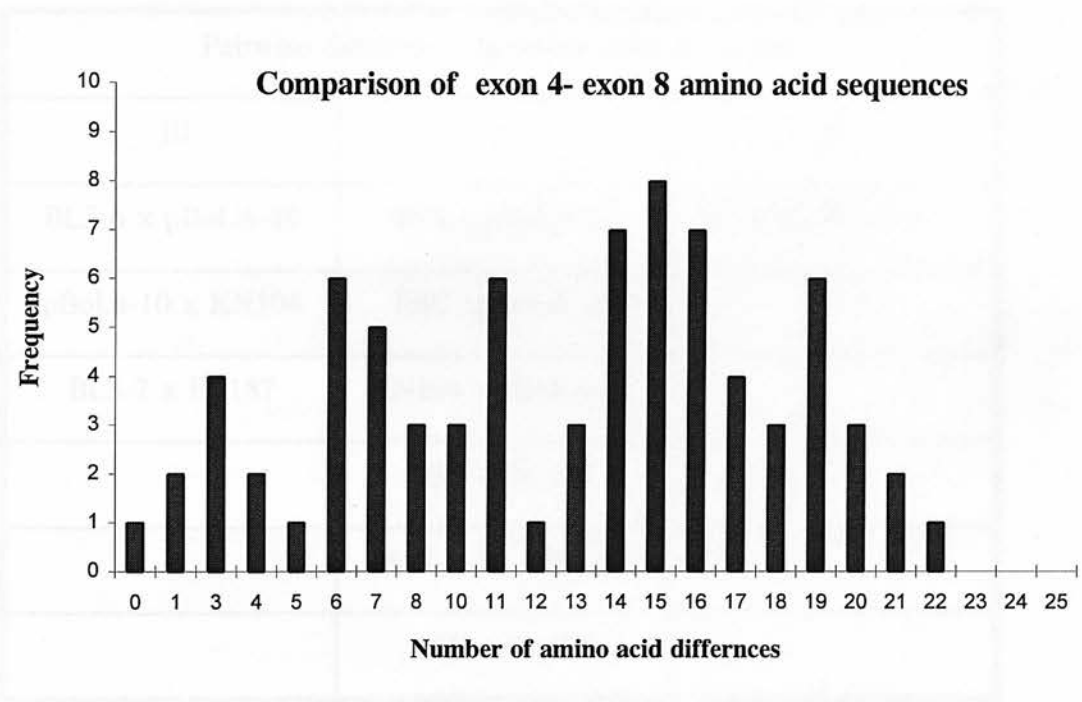


Table 2: Comparison of pairwise differences of amino acid sequences between the 1000 Genomes Project and the 1000 Genomes Project. The number of amino acid differences between two sequences is a measure of the genetic distance between the two sequences.

observation is supported from the topology of the phylogenetic tree (Fig 8.3) as these sequences were found on the outermost branch of the tree.

The pairwise comparisons of pBoLA-10 with pBoLA-4 and pBoLA-19 were in the range expected for interlocus comparisons as suggested from the earlier results. Also the difference between pBoLA-4 and pBoLA-19 pair showed a score of 13 which appears to be in the interlocus comparisons. This is also supported by the earlier analysis of 3'-ut and the differences in transmembrane and cytoplasmic domains of these sequences.

Pairwise differences in amino acids in number		
10	11	12
BL3-6 x pBoLA-19	BSA x pBoLA-19	Bl3-6 x pBoLA-4
pBoLa-10 x KN104	BSC xpBoLA-19	
BL3-7 x Bt 187	KN104 x pBoLA-19	
	BSN x Bt 187	
	pBoLA-19 x Bt 187	
	BSF x Bt 187	

Table 8.2 : Comaprison of pairwise differences of amino acids between BoLA class I sequences in the range of 10-12

8.6 Conclusion and problems

All of the methods used in the analysis of the three class I sequences suggested that the differences between the sequences were sufficient to be in the range expected for genes of different class I loci. The two A11 sequences (pBoLA-19 and pBoLA-4) appeared to be different genes encoding components of A11 haplotype. There may be variable numbers of expressed classical MHC class I loci in cattle, as is seen in between mouse strains (Steinmetz et al. 1982b; Weiss et al. 1984). This number could differ in different haplotypes as well as breeds, possibly as a result of gene duplication. It has been suggested that the genes of the BoLA class I loci are in linkage disequilibrium, so that the allo-antisera see the products of multiple genes, and thus are not gene locus-specific but haplotype specific. This is supported by the findings that the two genes encoding components of the A11 serological specificity were not differentiated by alloantisera although they were different genetically.

Previous analysis regarding the number of different class I loci in cattle has been primarily based on the cloning and sequencing of cDNA sequences from individual animal. This has produced evidence that at least three class I genes are transcribed in cattle (Garber et al. 1994). But all of the transcribed genes may not necessarily be expressed at the cell surface, so this approach alone is not sufficient to determine how many class I products are expressed.

All of the full length bovine sequences transmembrane regions of either 37 or 35 amino acids, with the exception of KN104 where the length is predicted to be 36 amino acids. Garber and co-workers (1994, suggested that these three patterns represent three different loci, however no published sequence except KN104 has revealed another 36 amino acid TM region, which suggests that KN104 is an

diverged allele which belongs in one of the other groups. KN104 was isolated from *Bos indicus* and this specificity is only found in African cattle, so it is not clear whether this is a difference between *B. taurus* and *B. indicus*. In addition, when the number of different sequences identified in individual animals is taken into account, it is clear that the products of more than one locus have a 35 amino acid TM e.g., the two sequences pBoLA-19 and pBoLA-4 have TM regions of 35 amino acids, but have locus specific features which make them likely to be two different genes on the same chromosome. These results indicate that assignment of sequences to loci will not be possible using the method of single cDNA isolation, but will require the identification and characterisation of the expressed products of known genes by transfection.

SUMMARY AND CONCLUSIONS

In cattle, the BoLA complex is presently defined serologically as a single allelic series of 52 internationally defined allo-specificities (Davies et al. 1994). Information regarding the genetic basis of these specificities was limited, until several class I sequences were published (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Garber et al. 1993, 1994; Sawhney et al. 1995). The number of expressed loci is important, as the class I gene products act in shaping the repertoire of T-cells available to the animal, hence partly determining its ability to respond to pathogens. Although there may be 20 or more genomic class I genes found in cattle by Southern analysis (Lindberg and Andersson, 1988), the number of expressed class I products in cattle is unclear. Biochemical (Joosten et al. 1992; Al-Murrani et al. 1993, 1994) and molecular biological studies (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Ellis et al. 1992; Garber et al. 1993, 1994) suggest that two or three MHC class I loci are transcribed. However, there has been no systematic attempt to confirm the number of expressed products. Most of these workers used a cDNA approach to analyse the number of expressed class I genes. It has been suggested that all of the genes which are transcribed may not necessarily be expressed on the cell surface, and thus are not likely to be functional antigen-presenting molecules (Ellis et al. 1995). The aim of this study was to investigate the expression and function of individual class I products, by the transfection and characterisation of bovine class I genomic and cDNA clones isolated from a single animal serologically typed as BoLA-A10/A11.

This study began with a group of 15 class I genomic clones, of which five representative clones were transfected into mouse L cells. Of the five different

clones transfected, only 19.1 and 4.2 showed expression at the L cell surface using the bovine monoclonal antibody IL-A88 (chapter 4). The transfectants obtained from both expressing clones were characterised serologically, biochemically and by cellular assays and both were found to encode the A11 serological specificity. Interestingly, the products of the 19.1 and 4.2 transfected class I genes were indistinguishable by isoelectric focusing, despite having clearly different nucleotide sequences in both the clones and in the transfected cells. These results suggested that both class I genes encoded a component of the A11 haplotype and, thus, represented the products of two different loci. These observations show that while serology may be of some use to study class I polymorphism, allo-antisera do not recognise the products of individual class I loci but instead recognise the products of multiple loci within a haplotype which further supports the idea that these loci are in linkage disequilibrium. Indeed, physical mapping studies by Bensaid et al. (1991) have shown tightly linked, expressed class I loci. From the BoLA workshops it has been suggested that the allo-antisera recognise allelic products (Spooner et al. 1979b; Bull et al. 1989; Bernoco et al. 1991). However, the observation that the 19.1 and 4.2 genes encode the same serological specificity, and the work of Al-Murrani and co-workers (1993) suggest that the allo-antisera are haplotype-specific rather than allele-specific. In order to extend the panel of allo-antisera and improve the ability to distinguish between products of different BoLA class I loci, it may be advantageous to use reciprocal immunizations between animals from genetically distant breeds. Animals from different geographical locations face distinct selection pressures, making them carry different allelic combinations, thus increasing the chance of producing sera that recognise the products of different genes (Kemp et al. 1988). Although it is generally expected that class I MHC molecules encoded by different loci have different isoelectric points (Yang et al. 1984), some overlap is

still observed. The predicted isoelectric points of the 4.2 and 19.1 gene products were very similar, 5.23 and 5.26 respectively. The results support the idea that bovine class I molecules encoded by different loci can have very similar isoelectric points. Indeed, similar pI values of 5.24 and 5.26 were predicted for the from BL3-6 and BL3-7 bovine class I cDNA sequences which have been suggested from different loci (Ennis et al. 1988).

Sequence data for about 500bp flanking the 5'-end of the class I gene (chapter 5) was obtained from clone 19.1, and a repetitive DNA element was discovered immediately upstream of the class I promoter (Sawhney et al. 1995). This clearly defines the start of the class I gene and may not be conserved of other class I loci. Such potentially locus-specific sequences will be invaluable for designing primers for amplification of the adjacent class I genes in a range of haplotypes, which could be further characterised for functional antigen presentation.

The lack of expression from the three phage clones 1.3, 15.2 and 17.3x suggested that these clones might contain truncated class I genes or pseudogenes. The three non-expressing clones did not express, even when subcloned in the expression vector pBoLA-21, suggesting that they are pseudogenes. However, these class I clones could be useful as probes to map the genes in and around the MHC region. Geraghty and co-workers (1992) reported more than twelve HLA class I pseudogenes. It was suggested that most of these pseudogenes evolved as a result of gene duplication events. The functional significance of these class I MHC genes which do not participate in antigen presentation remains unclear. It has been proposed that these genes may be a source of donor sequences for the generation of sequence variation in classical class I genes (Lawlor et al. 1990). An alternative

view is that these loci may represent relics of genes that were once used in antigen presentation and have been subsequently discarded, or the products of unsuccessful evolutionary experiments with antigen presenting genes (Klein and Figueroa, 1986; Howard, 1987). For example, in man HLA-AR alleles closely resemble alleles encoding HLA-A antigen-presenting molecules, although the presence of one or two deleterious mutations prevent these alleles being active in antigen presentation (Zemmour et al. 1990). This line of speculation argues that, depending on the antigen environment, there can be either positive or negative selective pressure on particular MHC alleles and loci. This suggests the MHC is a particularly dynamic system in which different genes and alleles are being brought into play and then discarded in the course of evolutionary time. This may, in a general sense, explain some of the unusual variability in the number of MHC genes.

It is also clear that the presence of these genes in the genome is not sufficient for their expression.

In order to isolate and characterise other functional class I genes from this animal, a cDNA approach was tried. The amplification of cDNA was achieved by using a selective primer based in the 3'-ut region of the class I gene and a conserved primer in the exon 2 area (chapter 7). A 1.2kb (exon 2 - 3'-ut) amplified cDNA was expressed after subcloning into the pBoLA-21 class I expression vector. Only one cDNA clone of the right size was isolated in these experiments, and most of the cDNA clones screened were shorter in length and were not further investigated. These cDNA clones might be truncated due to alternative splicing of transcripts or aberrant PCR. Ellis and co-workers (1992) have reported that some class I alleles were more prone to alternative splicing resulting in the removal of exon 7. It has been suggested that the length of internal exons play an important role in the splice site selection (Dominski et al. 1991). Internal exons less than 50bp are more likely to be involved in exon skipping. However, the shorter length transcripts were

truncated by 100-500bp. The transfectants obtained from the cDNA clone encoded a product with A10- specificity, which was confirmed by flow cytometry analysis with the A-10 specific monoclonal antibody IL-A34.

Sequence analysis of the three class I genes (chapter 8) suggested that all the three sequences are likely to be from different loci. The sequences on pBoLA-19 and pBoLA-4 encoded the same serological specificity (A11), and were very different in their transmembrane and cytoplasmic domains, and in the 3'-ut regions, strongly suggesting they are different genes of the A11 haplotype. The fact that both genes encode the same specificity also implies that they are different genes of the same haplotype. These observations suggest both A11 genes could have evolved by possible gene duplication mechanism as seen for the presence of multiple DQ genes in the class II region in some haplotypes of cattle (Marello et al. 1995). But there is no evidence for this since they are very distinct in sequence. It is also possible that there could be variation in the number of the expressed class I genes in different haplotypes as well as breeds. Variation in the number of expressed class I genes between horse breeds have been reported by (Lew et al. 1986). It was observed that many thoroughbred horses do not appear to carry a gene for the secreted class I molecule which is commonly found in most other horses. Variation in the number of class I genes between haplotypes has also been demonstrated in mice (Wroblewski et al. 1994), where D region can contain one and five loci. It has been shown that six distinct cDNA sequences, apparently from three loci, were expressed in heterozygous bull (Garber et al. 1994), in other haplotypes it appears that one or two genes may be transcribed (S.A. Ellis, unpublished observations). In this study three functional class I genes were isolated from the A10 and A1 haplotypes, but there may be more expressed class I genes in this animal. While two expressed A11 genes

were found the presence of two or three functional A10 haplotype genes cannot be ruled out, although only one expressed A10 gene was isolated. These observations are supported by the evidence that PBM from this animal were seen with all the three A10-specific mAbs, but isolated A10-encoded gene product was recognised by only IL-A34. So the presence of the other A10-like genes encoding component of A10 haplotype are likely to be expected. Also it was not possible to isolate allelic sequences from the A10 and A11 haplotypes so there is no evidence that the two haplotypes carry alleles of the same gene.

The results presented here demonstrate the feasibility of using transfection techniques and DNA sequencing to help define the number of functional class I MHC genes. Additionally, transfected cells are valuable tools for producing new serological reagents to MHC molecules, and for studying the role of individual MHC molecules in the restriction of CTL recognition of foreign antigens. These transfected cell lines expressing individual class I products could be useful for the determination of allele-specific class I peptide motifs, which have further application in the determination of T cell epitope for designing new vaccines. The peptide motifs for the two BoLA class I molecules A20 and A11 showed the presence of charged amino acid at the carboxy-terminus (Bamford et al. 1995; Hegde et al. 1995). This observation suggests the bovine TAP system is functionally similar to humans. The peptide motif for the A11 molecule has been suggested to be from the product of the pBoLA-19 gene (Hedge et al. 1995). Motifs the same as or similar to, for BoLA-A11 have been reported for HLA-B7, HLA-53, HLA-B*5101 and HLA-B*5102 (Rammensee et al. 1995). Sequence comparisons between these molecules and pBoLA-19 showed that key residues involved in accommodating motif residues P2 and P9 (ie 9, 24, 45, 67, 77, 116) share similar characteristics. The BoLA-A11

motif is consistent with this molecule assuming a similar conformation as human class I ABS. Not unexpectedly, therefore, the predictions on the peptide specificity of BoLA-A11 sequence is similar to the corresponding peptide motifs from this class I molecule. Since residue 67 (Tyrosine) in the pBoLA-19 sequence is predicted to block the B pocket, such that a residue with a small side chain is the most likely to be found in P2. In the peptide motif position 2 was occupied by Pro, which can kink the peptide to be accommodated in the B pocket (Hedge et al. 1995).

The expression vector pBoLA-21 successfully expressed both genomic and cDNA copies of class I genes. This system should also facilitate the functional characterisation of class I clones lacking exon 1 and 3'-untranslated regions which would otherwise difficult to express (eg. many of the published cDNAs). In addition, this system can be used to selectively amplify, clone and express different bovine class I genes for rapid characterisation of individual genes from a range of haplotypes.

Future studies

The results presented in this thesis show only the beginning of the correlation of serological specificities with the molecular characterisation of bovine MHC class I genes from the BoLA-A10 and A11 haplotypes. It would have been better if the genomic counterpart of the BoLA-A10 cDNA had been isolated allowing a clear comparison of genes from both haplotypes. The isolation of other clones expected to encode and express the products recognised by two A10-specific mAbs (IL-A7 and IL-A10) would help define the number of expressed genes from the A10 haplotype. Ideally isolation of overlapping genomic clones would help to identify how similar bovine MHC is that of mouse and man. Progress in the determination of the

range of functional bovine class I genes will require the use of homozygous animals, combining phylogenetic analysis of sequences with the identification and characterisation of expressed gene products in transfected cell lines. The transfected cell lines made during this project could be further used for the prediction of class I Motifs. Construction of pBoLA-21 allows rapid analysis of the other haplotypes without screening the libraries.

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Appendix 2A

TE buffer

10mM Tris pH8

1mM EDTA pH8

Electrophoresis buffer

TBE - Stock 10 x TBE

Per liter :

108g Tris

55g Boric acid

40ml 0.5 M EDTA pH8

TAE - Stock 50 x TAE

Per liter :

242g Tris

57.1ml glacial acetic acid

100ml 0.5 M EDTA pH8

30% Acrylamide stock solution

Acrylamide 29g

N, N-methylene bisacrylamide 1g

made to 100ml with autoclaved distilled water

Phage buffer.

This buffer is used for storage and dilution of bacteriophage λ stocks.

10 mM Tris HCl pH7.5

10 mM MgSO_4

0.01% gelatin

The phage buffer was autoclaved.

Cesium chloride solutions for step gradients prepared in phage buffer

Density	CsCl	Phage buffer(ml)
1.3	23.43	51.57
1.5	34.05	40.92
1.7	42.18	32.84

LB medium

Per liter :

Bacto-tryptone 10g

Bacto-yeast extract 5g

NaCl 10g

Adjust pH to 7.5 with sodium hydroxide and autoclave as 500ml stocks.

LB plates

The LB medium is made up according to the above formula and before autoclaving, one of the following was added (per liter) :

Bacto-Agar 15g (for plates)

Agarose 7g (for top agarose)

MOPS electrophoresis buffer 10x

0.2M MOPS

0.5M sodium acetate

0.01M EDTA

used at 1x

Appendix 2B

L cell growth medium

i) 10 ml of 10 x RPMI-1640

2 ml sodium bicarbonate 7.5% (to get pH6.8 to 7.2)

2ml L Glutamine

10ml foetal calf serum

0.5ml gentamycin

to 100ml with autoclaved distilled water

ii) Selection medium

The recipe is as above but 2ml of 50 x HAT is added to select for the transformants.

Phosphate-buffered saline (PBS)

Per 5 litres:

NaCl 42.5g

N_2HPO_4 5.35g

NH_2PO_4 2.55g

made up in distilled water and autoclaved.

Eosin stain (2', 4', 5', 7',- tetrabromofluorescein) (Sigma).

5% eosin in 1 x HBSS

Stored at 4°C. Stable for 1 week.

Fixing solution

40ml formaldehyde

60ml 0.9% NaCl

5ml 0.15 M Na_2HPO_4

Table: The list of mAbs used during this project and their specificity.

mAb	Specificity	working dilutions	Reference
IL-A88	Bovine MHC class I	1:1000	Bensaid et al. 1991
IL-A12	CD4	1:1000	Howard et al. 1991
IL-A51	CD8	1:500	Ellis et al. 1986
IL-A7	BoLA- A10	1:1000	Toye et al. 1990
IL-A10	BoLA-A10	1:1000	<i>do</i>
IL-A34	BoLA-A10	1:1000	<i>do</i>
W6/32	HLA class I	1:500	Brodsky and Parham, 1982

LIST OF PUBLICATION FROM THESIS

Sawhney, S.M.S., Hasima, N.N., Glass, E.J., Al-Murrani, A.W.K., Nichani, A.K., Spooner, R.L., Willams, J.L., and Russell, G.C. Transfection, expression, and DNA sequence of a gene encoding a BoLA-A11 antigen. *Immunogenetics* 41: 246-250, 1995

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BRIEF COMMUNICATION

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Transfection, expression, and DNA sequence of a gene encoding a BoLA-A11 antigen

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Cattle major histocompatibility complex [(MHC) (BoLA)] class I molecules are heterodimeric glycoproteins which present endogenous antigenic peptides to CD8⁺ T lymphocytes, initiating a cellular immune response. The MHC-encoded heavy chains are highly polymorphic and, in cattle, have been characterized mainly by using allo-antisera raised by reciprocal calf/dam immunisations (Spooner et al. 1978). This has enabled the identification of about 50 serological specificities, most of which behave as alleles of a single highly polymorphic class I locus (Davies et al. 1994). However, evidence from biochemical (Joosten et al. 1992; Al-Murrani et al. 1993, 1994) and molecular biological studies (Ennis et al. 1988; Brown et al. 1989; Toye et al. 1990; Bensaid et al. 1991; Ellis et al. 1992; Garber et al. 1993, 1994) suggest that more than one BoLA class I locus is expressed. These loci are apparently in linkage disequilibrium (Al-Murrani et al. 1993), making them difficult to distinguish by conventional methods. In order to investigate the expression and function of individual class I locus products, we are correlating BoLA class I gene sequences with the expressed products by the transfection and characterization of genomic class I clones. Shotgun transfection and expression of BoLA class I molecules has been described previously, but the genes involved were not isolated (Toye et al. 1990). In this paper we report the isolation, DNA sequencing, transfection, and expression of a genomic clone encoding a BoLA-A11 determinant from an animal expressing A10 and A11 serological specificities.

A genomic library was prepared in the lambda vector EMBL3, using peripheral blood mononuclear cell (PBM) DNA from an animal with A10 and A11 serotypes. The library was screened by hybridization with a ³²P-labeled cattle MHC class I cDNA clone, pBoLA-1 (Brown et al. 1989). A total of 15 independent clones were selected from the library, which hybridized with both 5' and 3' ends of pBoLA-1 and were considered candidates for full-length genes. These clones were tested for expression by calcium phosphate mediated transfection into murine L cells, as described by Wigler and co-workers (1979), with the thymidine kinase (*tk*) gene as a selectable marker (Hasima 1992). Expression was examined by flow cytometry (FACS), using monoclonal antibody (mAb) IL-A88, which recognizes a monomorphic, non-conformational epitope on BoLA class I heavy chains (Toye et al. 1990). Only one clone, 19.1, showed significant levels of expression of a BoLA class I product. Three rounds of FACS sorting produced a population in which all of the cells expressed a 19.1 product. This purified population of transfected cells was characterized by serological, cellular, and biochemical methods.

The identity of the expressed antigen was initially investigated by using a BoLA class I micro-lymphocytotoxicity test (Spooner et al. 1978) with allo-antisera routinely used to define the A11 specificity (Davies et al. 1994). The transfected L cells expressing the product from clone 19.1 were lysed by three A11-specific allo-antisera: Ed73, Ed102, and Ed110, but were not killed with a fourth A11-specific allo-antiserum, Ed76. Control L cells were not killed with any of the A11 sera, and neither the control nor the transfected L cells were killed with sera defining other BoLA specificities. The killing of the transfected cells required the use of higher concentrations of the allo-antisera than for normal cattle PBM. This may reflect the degree of expression of the transfected gene, or may indicate that L cells are more robust than cattle PBM.

Functional analysis of the product expressed by the clone 19.1-transfected cells was done using anti-A11 and anti-A10 allo-reactive cytotoxic T lymphocytes (CTL). The CTL lines were generated in vitro as described previously

The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases, and have been assigned the accession numbers X82671–X82675

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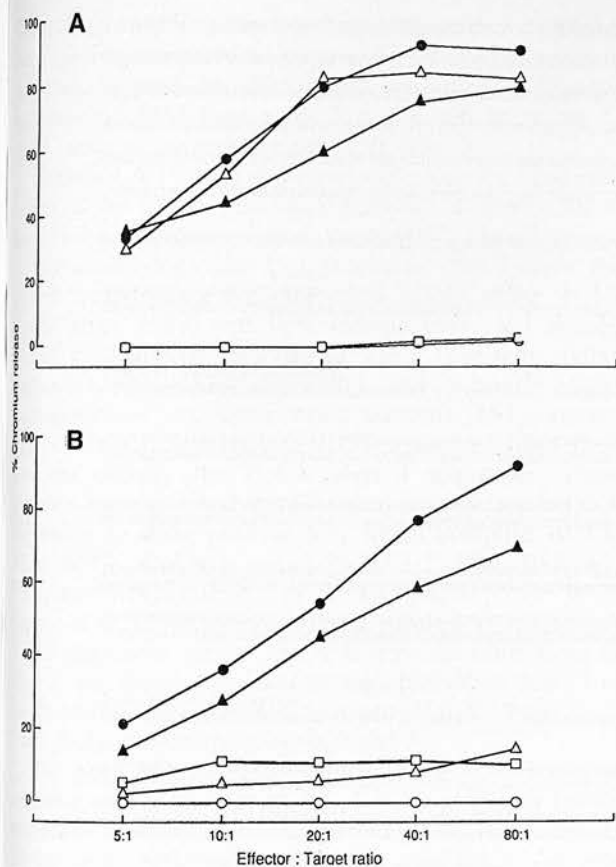


Fig. 1 BoLA class I-specific ^{51}Cr -release cytotoxicity assay of clone 19.1-transfected cells with: **A** anti-A11 specific; and **B** anti-A10 specific CTL, performed essentially as described previously by Spooner and co-workers (1987). *Theileria annulata*-infected lymphoblastoid cell lines (Ta) were used as target cells for establishing specificity of effector cells. Transfected and control L cells were harvested with 0.02% ethylenediaminetetraacetate 16 h before the assay and incubated overnight in DMEM medium in non-adherent polystyrene petri dishes (Townsend et al. 1984). The panel of target cells were as follows: **A** ● Ta from the donor animal, BoLA A10/A11; ▲ Ta, BoLA A11/-; □ Ta, BoLA A6/A31; △ Clone 19.1 transfected L-cells; ○ Control L cells. **B** ● Ta from the donor animal, BoLA A10/A11; ▲ Ta, BoLA A10/A11; □ Ta, BoLA A3/A13; △ Clone 19.1 transfected L cells; ○ Control L cells

(Spooner et al. 1987), except that after four rounds of restimulation, CD4^+ cells were depleted by complement mediated lysis, using the mAb cc8 (Howard et al. 1989) to enrich cell populations for CD8^+ cytotoxic T cells. The cell lines produced were 97% CD8^+ as assessed with mAb IL-A51 (Ellis et al. 1986). The A11-specific CTL line killed L cells transfected with clone 19.1 and cells from the donor animal equally well (Fig. 1A), whereas the transfected cells were not recognized by the A10-specific CTL line (Fig. 1B). Target cells expressing other BoLA class I antigens and control L cells were not killed by either CTL line. Recognition of the transfected cells by the CTL lines could be inhibited by treating the target cells with the MHC class I-specific mAbs IL-A88 and W6/32 (A. K. Nichani, unpublished data).

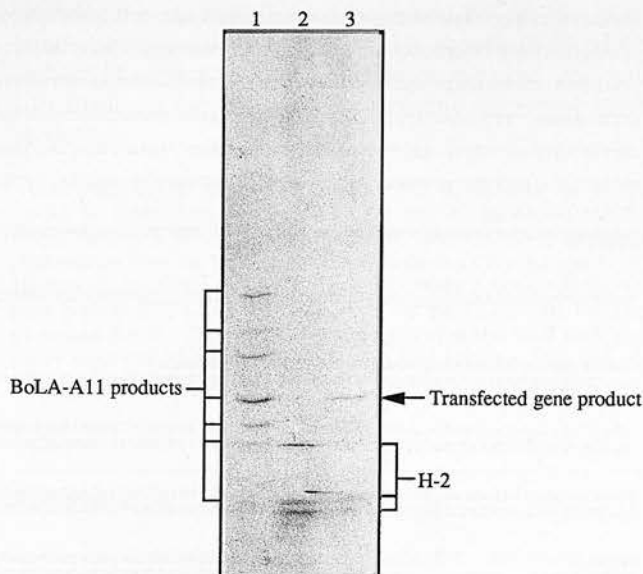


Fig. 2 Iso-electric focusing of class I antigens. MHC class I antigens were analyzed by 1D-IEF essentially as described by Joosten and co-workers (1988), except that for the control and transfected L cells, 10^8 cells were labeled for 6 h with 300 μCi of ^{35}S -methionine (Amersham) for each precipitation. The gel shows ^{35}S -labeled molecules immunoprecipitated with the allo-antiserum Ed102 which is specific for the BoLA-A11 haplotype from: lane 1 PBM from an A10/A11 animal; lane 2, control L cells; and lane 3, L cells transfected with clone 19.1

Biochemical analysis of the clone 19.1 gene product was done by 1D-IEF as described by Joosten and co-workers (1988). The A11-specific allo-antiserum Ed102 was used to immunoprecipitate cell-surface molecules from A11+ PBM, Ltk $^-$ control cells and the clone 19.1-transfected cells after metabolic labeling with ^{35}S -methionine. The banding pattern of the PBM sample contained several charge variants (Fig. 2, lane 1), as reported previously (Al-Murrani et al. 1993). Although the serum Ed102 cross-reacted with H2 molecules expressed by the control Ltk $^-$ cells (Fig. 2, lane 2), it immunoprecipitated a single additional molecule from the transfected L cells (Fig. 2, lane 3). This transfectant-specific band had the same iso-electric point as one of the A11 molecules from PBM. This adds weight to the serological and cellular identification of the 19.1 gene product as a component of the A11 haplotype.

The lambda EMBL3 clone 19.1 contained a 21 kilobase (kb) DNA insert with all of the pBoLA-1 hybridization localized to a 1 kb *Sal*I-*Bam*HI fragment and the adjacent 7 kb *Bam*HI fragment (Hasima 1992). These fragments were gel-purified and subcloned into pBR322 to form pBoLA-17 and pBoLA-18, respectively, from which the original arrangement of fragments was reconstructed in pBoLA-19. To ensure that pBoLA-19 carried the entire class I gene from phage clone 19.1, it was co-transfected with the *tk* gene into Ltk $^-$ cells. After HAT selection, over 90% of the surviving transfectants expressed a cattle class I molecule as detected by FACS analysis, confirming the integrity of the gene on pBoLA-19. Both phage- and



plasmid-derived transfectants were used in subsequent analyses, with identical results.

DNA from plasmids pBoLA-17, pBoLA-18, and pBoLA-19 was sequenced by the dideoxy chain-termination method (Sanger et al. 1977), with modified T7 DNA Polymerase (Sequenase; Amersham, Amersham, UK). For initial sequencing we used primers designed from conserved regions of the published cattle class I cDNA sequences (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Ellis et al. 1992; Garber et al. 1993) and pBR322-specific primers flanking the cloned fragments.

Fig. 3 The nucleotide sequence of the class I gene from the lambda clone 19.1. Intron sequences are given in *lowercase*. The translated amino acid sequences of the exons are given in *three-letter code* above the DNA sequence. Two possible translation initiation codons are shown in *italics*. The region equivalent to the class I regulatory complex (CRC) sequence in *HLA* genes is shown in **bold type**, and promoter sequences are underlined and indicated as follows: CAAT, CCAAT-box; TATA, TATAA box. Predicted polyadenylation sequences are in **bold italics**. Restriction enzyme sites are overlined and labeled as follows: Bam, Bam HI; Pvu, Pvu II. The estimated sizes in bp of unsequenced regions are given at their appropriate positions in *ellipses* (...)

Extension primers, based on the sequence data obtained, were used to complete the sequences of all of the exons in the class I gene on clone 19.1, including contiguous segments of 1034 base pairs (bp) and 886 bp at the 5' and 3' ends of the gene, respectively (Fig. 3).

The pBoLA-19 exon sequences were combined and used to search the EMBL nucleotide sequence database. All of the similar sequences found were MHC class I genes, clearly identifying clone 19.1 as a cattle class I clone, the closest match being with the cattle cDNA clone BL3-7 (Ennis et al. 1988) with 96% identity over 1451 aligned bases. Phylogenetic analysis of class I protein coding sequences, analysis of the length and sequence of the transmembrane and cytoplasmic domains, and comparisons of the 3' untranslated (UT) sequences have been used to classify the BoLA class I sequences. These methods have assigned the published BoLA class I cDNA sequences to three putative loci which comprise BL3-6, BSA, BSC, pBoLA-1 and A10; BL3-7, BSF, BSN and BSX; and KN104 (Ennis et al. 1988; Ellis et al. 1992; Garber et al. 1994). Analysis of the pBoLA-19 coding and 3' UT sequences suggest that it is from the same locus as BL3-7, and this is supported by the observation that 21 of the 26 amino acid differences between pBoLA-19 and BL3-7 are in the polymorphic exons 2 and 3.

The pBoLA-19 sequences upstream of exon 1 can be split into two distinct areas based on similarity to known sequences. The region from position 1 to about 630 is similar to the Art2 repetitive element in the cattle lysozyme gene (Irwin et al. 1993), suggesting that this region may be part of a related repetitive DNA sequence. The remainder of the 5' flanking region is homologous with MHC class I promoters, containing an identifiable class I regulatory complex (CRC) and promoter elements (Fig. 3). In humans, the CRC sequences are highly conserved between alleles of a single locus (96%–99% identity), but are less well conserved between loci (78%–89% identity; Cereb and Yang 1994). The degree of identity between the CRC of pBoLA-19 and the cattle promoter sequences BTMHCI (J. S. Harms, unpublished data; accession L19193) and BoLAenh-9 (Harms and Splitter 1994) is 92% and 86%, respectively, suggesting that these promoters may be from different class I loci. Interestingly, the exon 1 sequence of BTMHCI is identical to that of the cDNA BL3-6, while pBoLA-19 appears to be allelic with BL3-7.

By transfecting a clone containing a well-characterized single gene, we were able to assign a sequence to the A11 gene and identify the product it encodes. Importantly, the integrity of this class I gene product is maintained in the transfectant, as determined by serological, functional, and biochemical analysis. Iso-electric focusing shows that only one BoLA class I product is immunoprecipitated from the transfectant, whereas the A11 allo-antisera recognize several molecules from PBM. Thus, allo-antisera recognize several gene products associated with individual haplotypes. Furthermore, the IEF patterns assigned to serologically defined haplotypes by family studies are consistent regardless of breed or origin of the typed cattle (Joosten et al. 1988; Al-Murrani et al. 1993; Davies et al. 1994),

implying that the cattle class I loci are in linkage disequilibrium. The approach described in this paper will allow the number of expressed class I loci to be resolved, and will also facilitate the functional analysis of individual locus products.

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The cattle major histocompatibility complex (BoLA) has been mainly characterized by using serological repertoires which have enabled the identification of a large number of polymorphic loci. However, the identification of a large number of highly polymorphic loci (Davies *et al.* 1994). We are transfecting genomic clones and cDNA clones from a animal with A10(A1) serological specificities to correlate BoLA class I gene sequences with the transfection of bovine MHC class I genes based on a novel plasmid expression vector. The plasmid vector pBoLA-1 (Genbank) carries the 5'- and 3'-ends of a characterized genomic class I clone (pBoLA-1) (Sawhney *et al.* 1995), flanking a unique *Eco* I site which is used for the subcloning of PCR-amplified class I gene fragments. This system has been tested by subcloning the coding region from two expressible genomic phage clones and two cDNA clones. Mouse 1 cell transfectants expressing bovine class I molecules were obtained from both the reconstructed genomic clones, and from the cDNA clones.

	Intron 1	Exon 2
BL3-7
BSP
BSN
BSA
BSA
W10
phoA
BSB
BSB
phoA-19

glutamatec dipeptidase ONTOGENIC STRAIN



A fragment containing the region between exon 2 and the 3'-untranslated region was amplified from the genomic class I clones. The primers were based on conserved sequences in exon 2 (forward primer A, Fig. 1) and in the 3'-untranslated region (reverse primers B and E, Fig. 2) of the published bovine class I sequences (Davis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Barber et al. 1993, 1994, 1997, 1998; Swinney et al. 1995). In addition to the conserved sequences from exon 2, primer A also included 10 bases of flanking intron sequence to allow correct splicing of the vector-occluded first exon, and a terminal *Xba*I site for cloning (in italics). The reverse primer B was designed for amplification of all possible class I sequences whereas primer E had 15bp of sequence which was missing in the pB60A-19 and BL3-7 class I sequences, and was expected to give locus-specific amplification. By using primers A and B, a 3.2 kb fragment was cloned from the genomic clones. (see Fig. 5)

The diagram illustrates the construction of pBlot-A21 and pBlot-A20 c1' from pBlot222. The process involves restriction enzyme digestion with PstI and EcoRI, followed by ligation with a linker and PCR amplification.

- pBlot222** is a circular plasmid with a 3.0 kb insert. It contains a PstI site (P) and an EcoRI site (E).
- Digestion with PstI and EcoRI results in a 3.0 kb fragment and a 1.0 kb fragment.
- The 3.0 kb fragment is ligated with a linker (1.0 kb) to form pBlot-A21.
- pBlot-A21 is then digested with PstI and EcoRI to form pBlot-A20 c1'.
- pBlot-A20 c1' is PCR amplified to yield a 1.0 kb fragment.

To generate the expression vector, 1.2 kb *Sal I*-*Eag I* fragment containing 5'-flanking sequences, the class I promoter, and 120 base pairs (bp) of 5'-untranslated region (pBlot A-19) in pCR222 to make pBlot A-20. Next, a 450 bp *Pvu II* fragment from pBlot A-19 containing the 3'-untranslated region and positive polyadenylation sequences was subcloned into pBlot A-20 (Fig. 3). Inverse PCR using primers C + D was used to produce a 4,440 acceptor fragment from pBlot A-20. Primer C was placed in intron 1, 440 bp upstream of the *Eag I* site, and primer D was the reverse complement of primer B. The PCR fragment amplified from pBlot A-20 was self-ligated to make pBlot A-21 which acts as a receptor for class I sense fragments (Fig. 4).

Diagram of the TA vector plasmid. The plasmid is circular and labeled "TA vector clone". It contains several restriction sites: HindIII, KpnI, XbaI, SalI, BamHI, SmaI, PstI, and EcoRI. An arrow indicates the direction of "SmaI digestion".

cloning of *Eco*I fragment
in plid.Δ-21

Figure 6.

2.1 kb

Diagram of a circular plasmid map. The plasmid is labeled '2.1 kb'. It contains several restriction sites: EcoRI (E), XbaI (X), SalI (S), PstI (P), BamHI (B), KpnI (K), SmaI (S), and NotI (N). A shaded segment represents the cloned EcoRI fragment. An arrow indicates the cloning site.

2

The selection of 'primer sites in the described parts of the gene ensured that the primers could be used to amplify both genomic and cDNA copies of class I genes. To test the reconstruction system for cDNA, a bovine class I cDNA fragment containing exon 2, 3'-ut was amplified from a bovine cDNA library (primers A1 and B; Brown et al., 1989) which was inserted at both 5' and 3' ends of the plasmid. Primers A and E contained terminal *Eco*I sites for direct cloning into amplified pBiol-A1 cDNA into pBiol-A21 to form pBiol-A20. The reconstructed pBiol-A20 cDNA expressed a detectable bovine class I product on transfection, suggesting the correct splicing of the hybrid gene. After successful transfection, expressing the pBiol-A1 cDNA, the gene-specific (primer E) based on an in vivo specific sequence of cDNA from total cellular RNA prepared from PBMC with an A1/A10A11 specificity (Fig. 8). The 1.2 kb amplified cDNA product was digested with *Eco*I to generate compatible ends for direct cloning into pBiol-A21 to form pBiol-A10. The transfectants obtained from the reconstructed cDNA clone pBiol-A10 expressed bovine class I molecules on mouse L cell surface.

This system will allow the rapid and direct cloning of class I genes in expressible form from either genomic DNA or cDNA.

Potential gene-specific primers, based in the 3'-untranslated regions of class I genes, can be used to selectively amplify, clone and express different bovine class I genes in this system.

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Figure 8.

A novel expression vector for transfection of bovine MHC class I genes

(Major Histocompatibility Complex; gene expression; polymerase chain reaction; RT-PCR)

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Abbreviations: MHC, Major Histocompatibility Complex; PCR, polymerase chain reaction; RT-PCR, reverse-transcription-polymerase chain reaction; bp, base pairs; kb, kilobase pairs; mAb, monoclonal antibody; PBM, peripheral blood mononuclear cells.

SUMMARY

A vector is described for the rapid expression of genomic or cDNA copies of bovine MHC class I genes in transfected mammalian cells. Class I gene fragments are amplified by the polymerase chain reaction using primers in conserved regions of exon 2 and the 3'-untranslated region of the gene, and can be subcloned directly into the expression vector pBoLA-21, which contains the necessary 5'- and 3'- sequences for correct expression. The vector was tested by subcloning and expressing genomic and cDNA clones, and by selectively amplifying, cloning and expressing a gene encoding one component of the BoLA-A10 haplotype from crude cDNA.

INTRODUCTION

The bovine major histocompatibility complex (BoLA) encodes a range of cell surface glycoproteins which mediate the presentation of antigenic peptides to T cells for immune surveillance. Serological analysis of BoLA class I molecules has identified about 50 serological specificities, most of which behave as alleles of a single highly polymorphic locus (Davies et al. 1994). Biochemical and molecular biological analyses, however, have provided evidence that BoLA class I molecules are the products of three or more genes (Al-murrani et al. 1994; Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Garber et al. 1993, 1994; Sawhney et al. 1995) which may be tightly linked. In order to correlate BoLA class I gene sequences with their expressed products we are cloning and transfecting class I genes from an animal which expresses A10/A11 serological specificities. As part of this work, we have developed a system for the transfection of bovine MHC class I genes based on a novel plasmid expression vector. The plasmid vector (pBoLA-21) carries the 5'- and 3'-ends of a characterised genomic class I clone (pBoLA-19; Sawhney et al. 1995), flanking a unique *Eag* I site which is used for the subcloning of PCR-amplified class I gene fragments. This system has been tested by subcloning the coding regions from two expressible genomic phage clones and one cDNA clone.

RESULTS AND DISCUSSION

(a) Primer selection and amplification of the coding fragment

In order to amplify MHC class I genes from genomic or cDNA, primer sites were selected in regions conserved in all of the published bovine class I sequences (Ennis et al. 1988; Brown et al. 1989; Bensaid et al. 1991; Garber et al. 1993, 1994; Sawhney et al. 1995). Since the majority of these sequences represent truncated cDNA clones, the 5'-end primer site was located at the start of exon 2, while the 3'-end primer was located within the 3'-untranslated region (Fig. 1). Amplification using these primers yields a fragment of 3.2kb from genomic class I genes and 1.2kb from cDNA.

(b) Construction of the class I expression vector, pBoLA-21

To allow the PCR-amplified gene fragments to be expressed, a pBR322-derivative vector was constructed in which the 5'- and 3'-ends of the genomic class I gene on pBoLA-19 (Sawhney et al. 1995) flanked a unique *Eag* I restriction site into which the PCR products could be cloned (Fig. 2). By providing the promoter, exon 1, and polyadenylation sequences on the expression vector pBoLA-21, it was hoped that this vector could be used to characterise the products of class I genes amplified from crude genomic or cDNA, or from truncated genomic or cDNA clones.

(c) Reconstruction of genomic class I clones in pBoLA-21

The PCR primers for amplifying bovine MHC class I gene fragments and the expression vector pBoLA-21 were tested using two positively-expressing genomic clones (19.1 and 4.2; Sawhney et al. 1995). The 3.2kb class I gene fragments amplified from the genomic class I clones with primers A and B were subcloned into the TA cloning vector pCRII (Invitrogen). This allowed the PCR products to be analysed and subcloned into pBoLA-21 using *Eag* I sites in primer A and in the TA vector polylinker. Both reconstructed clones were found to express a detectable cell-surface class I product after transfection into mouse L cells, confirming the validity of the approach. Fig. 3 shows histogram FACS analysis of the 19.1

reconstructed clone using the anti-bovine pan-class I mAb IL-A88. About 90% of the transfected cells expressed bovine class I molecules.

(d) Subcloning and expression of cDNA clones in pBoLA-21

The selection of primers sites in transcribed parts of the gene meant that the primers could be used to amplify both genomic and cDNA copies of class I genes. To test pBoLA-21 for cDNA expression, a 1200bp fragment containing was amplified from a truncated bovine class I cDNA clone (pBoLA-1; Brown et al. 1989), using primers A and E (Fig. 1). These primers contained terminal *Eag* I sites for direct cloning of the amplified pBoLA-1 cDNA fragment into pBoLA-21 to form pBoLA-30, and primer E was based on a potentially locus-specific segment of the pBoLA-1 3'-untranslated sequence which is conserved in BL3-6, W10 and KN104, but was absent from pBoLA-19 and BL3-7. The reconstructed pBoLA-30 cDNA expressed a detectable bovine class I product on transfection, suggesting that the hybrid transcript was correctly spliced and expressed.

After successfully expressing the pBoLA-1 cDNA, the potentially locus-specific primer (primer E; Fig. 1) based in the 3'-ut sequence of pBoLA-1-like sequences was used in an attempt to selectively amplify an A10 class I cDNA from total cellular RNA prepared from PBM with A10/A11 specificity. The 1.2kb amplified cDNA product was digested with *Eag* I and cloned into pBoLA21 to form pBoLA-10. The reconstructed cDNA clone pBoLA-10 expressed a bovine class I molecule on the surface of transfected L cells. The serological specificity of the product expressed by the pBoLA-10 transfected cell line was examined using IL-A88 and three A10-specific mAbs IL-A7, IL-A10 and IL-A34 (Toye et al. 1990). The transfectant reacted positively with IL-A88 and IL-A34 but not with IL-A7 or IL-A10 (Fig. 4), suggesting that pBoLA-10 encodes a component of the A10 specificity, and that the A10 specificity comprises the products of more than one gene.

(e) Conclusions

The primers and vector described here allows the rapid and direct cloning of class I

genes in expressible form, from genomic or cDNA. They will also facilitate the functional characterisation of class I clones lacking exon 1 or 3'-untranslated regions which would otherwise be difficult to express (*e.g.* many of the published cDNAs). Potentially gene-specific primers, based in the 3'-untranslated regions of class I genes, may also be used to selectively amplify, clone and express different bovine class I genes in this system.

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FIGURE LEGENDS

Fig.1. Multiple nucleotide sequence alignments illustrating the selection of primers for amplification of MHC class I gene fragments. In the alignments residues identical to the pBoLA-19 sequence are represented by *dashes* (-), regions of sequence which were not determined are shown by *dots* (.), and gaps inserted to maximise similarity in the alignment are shown by *carets* (^). The sequence of primer A and the reverse complement of primers B and E are given beneath the alignments. Sequences in *italics* in primers A and E are restriction enzyme sites included to facilitate subcloning of the PCR products, and the following ambiguity codes represent degenerate positions in the primer sequences: S, G or C; M, A or C; Y, C or T; and W, A or T. The primers were based on sequences conserved in exon 2 and in the 3'-untranslated region of the published bovine class I sequences. **(A)** The forward primer A included 10 bases of flanking intron sequence, in addition to the conserved sequences from exon 2, to allow correct splicing of exon 2. **(B)** Reverse primer B was designed for amplification of all possible class I sequences whereas primer E contained a 15bp region which was missing from the pBoLA-19 and BL3-7 sequences, and was expected to give locus-specific amplification.

Conditions for PCR amplification were as follows: denaturation at 94°C for 30 sec, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 minutes. The last extension step was for 5 minutes at 72°C. The reaction buffer contained 10mM KCl, 10mM NH₂SO₄, 20mM Tris-HCl (pH8.8), 2mM MgSO₄, 0.1% Triton X-100, 200µM of dNTPs and 20 pmol of each primer. To achieve amplification of fragment size larger than 3kb and also to ensure the accuracy of replication, addition of vent DNA polymerase to Taq DNA polymerase at a 1:10 ratio was used.

Fig. 2. The expression vector pBoLA-21 was constructed as follows: **(A)** a 1.2kb *Sal* I-*Eag* I fragment containing the class I promoter and exon 1 was subcloned from pBoLA-19 into pBR322. Next, a 450bp *Pvu* II fragment from pBoLA-19 containing most of the 3'-untranslated region and polyadenylation sequences was added to make pBoLA-20. **(B)** Inverse PCR using primers C and D (marked by arrows) was then used to amplify a 4.4kb receptor fragment from pBoLA-20. Primer C was based on intron 1 sequences flanking the *Eag* I site, and primer D was the reverse complement of primer B. Finally, the PCR fragment amplified from pBoLA-20 was self-ligated to make pBoLA-21.

Restriction enzyme sites, and important features of the sequences are shown as follows: S, *Sal* I; E, *Eag* I; P, *Pvu* II; Pr, class I promoter region; Ex1, pBoLA-19 exon 1; Ap^R, Ampicillin resistance gene; Tc^R, Tetracycline resistance gene; ori, pBR322 origin of replication.

Fig. 3. Fluorescence-activated cell scan (FACS) analysis of bovine class I molecules expressed on the surface of transfected cells. Cells were labelled with the anti-bovine class I monoclonal antibody IL-A88, followed by a fluorescently-labelled anti-mouse IgG secondary antibody. After washing, the degree of cell fluorescence (ordinate) is plotted against cell number (abscissa) for both the negative control (untransfected L cells, open curve) and for the reconstructed pBoLA-19 class I gene in pBoLA-21 (closed curve).

Fig. 4. FACS analysis of the pBoLA-10 transfected cell line with **(A)** IL-A7, **(B)** IL-A10, **(C)** a murine monoclonal antibody specific for a non-polymorphic determinant on bovine MHC class I (IL-A88), and the BoLA-A10 specific mAbs and **(D)** IL-A34. Cells were labelled with the class I-specific primary antibody, followed by the appropriate fluorescent secondary antibody, and were analysed as described in Fig. 3.

A

	Intron 1		Exon 2	
BL3-7	-----	-----
BSF	-----	-----
BSN	-----	-----
BL3-6	-----	-----
BSA	-----	-----
W10	-----	ga-----
pBoLA-1	-----	ga-----
BSC	-----	-----
KN104	-----	-----
pBoLA-19	gtctcaccct	cttgccccag	GCTCCCACTC	CCTGAGG
	GAGGTCCGGCCG	CTTGCCCCAG	GCTCCCACTC	SMTGA
		Primer A		

B

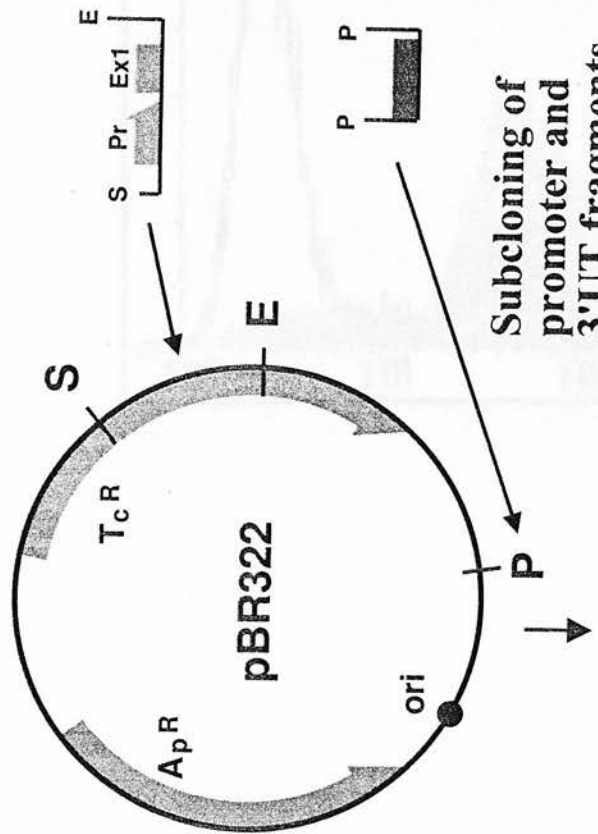
```

3'-untranslated region

W10  ----TGCTG CCCTTCCCCA C---GT--- ----- ---T---
BL3-6 ----TGCTG CCCTTCCCCA C---GT--- ---G--- ---T---
pBoLA-1 ----TGCTG CCCCTCCCCA CCCTGAC-.. ..... ---T---
KN104 ----TGCTG CCCCTCCCCA C---^-- ---T---
BL3-7 ----^----- G---^-- -----
pBoLA-19 CCCAC^-----C ATGCC^CCC TCCCCACCCT GACCTGTGTT CTCCTCCC

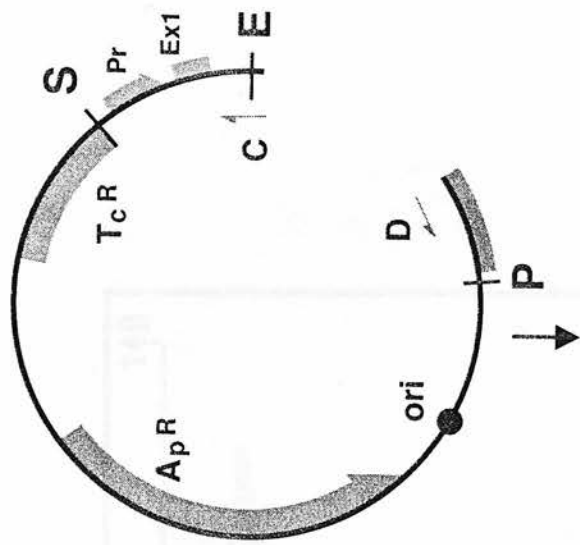
CCCACTGCTG CCCYTCCCCA CGGCCGCCCC
Primer E CCC TCCCWACCCT GACCGGTGTT CTCYTCC
Primer B

```



Subcloning of
promoter and
3'UT fragments

PCR amplification
of vector with
primers C and D



Blunt-end ligation
of PCR product

cloning site for
class I gene
fragments

